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THE JOURNAL OF APPLIED BACTERIOLOGY

(Formerly Proceedings of the Society for Applied Bacteriology)

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Greer, S. B. Growth inhibitors and their antagonists as mutagens and antimutagens in *Escherichia coli*.
Chang, S. L. Cultural, cytological and ecological observations on the amoeba stage of *Naegleria gruberi*.
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Banic, S. Isolation of Lac+ mutants from a Lac− strain of *Escherichia coli*, by the replica plating technique.
Bradley, S. G. Mechanism of "vegetative hybridization" in Streptomyces.
Jones, A. S., Rizvi, S. B. H. & Stacey, M. The phosphorus-containing compounds of Gram-positive and Gram-negative organisms in relation to the Gram staining reaction.
Pontieri, G. & Schiano, S. Observations upon the growing points in normal and filamentous *Bacillus megaterium*.
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Vanek, Z., Dolezilova, L. & Rehacek, Z. Formation of a mixture of antibiotic substances, including antibiotics of a polyene character, by strains of Actinomycetes freshly isolated from soil samples.
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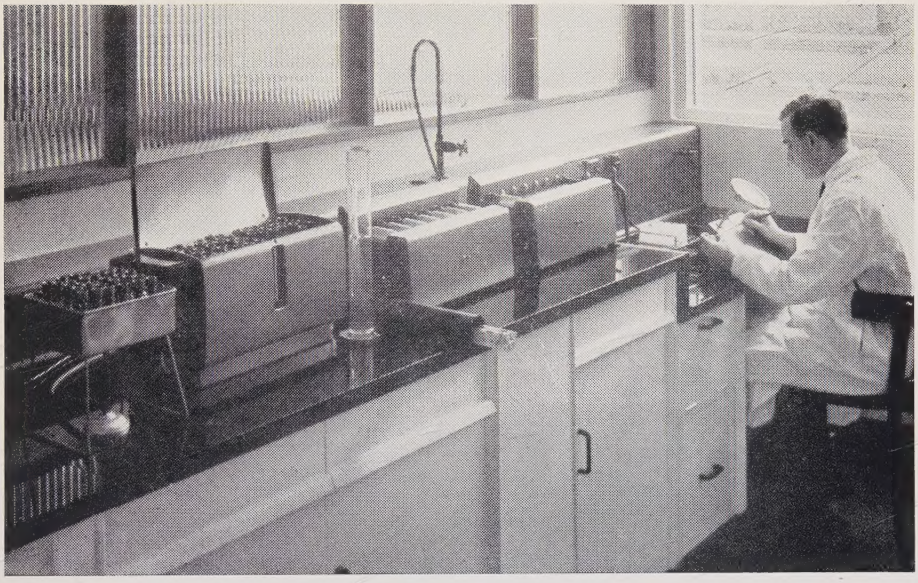

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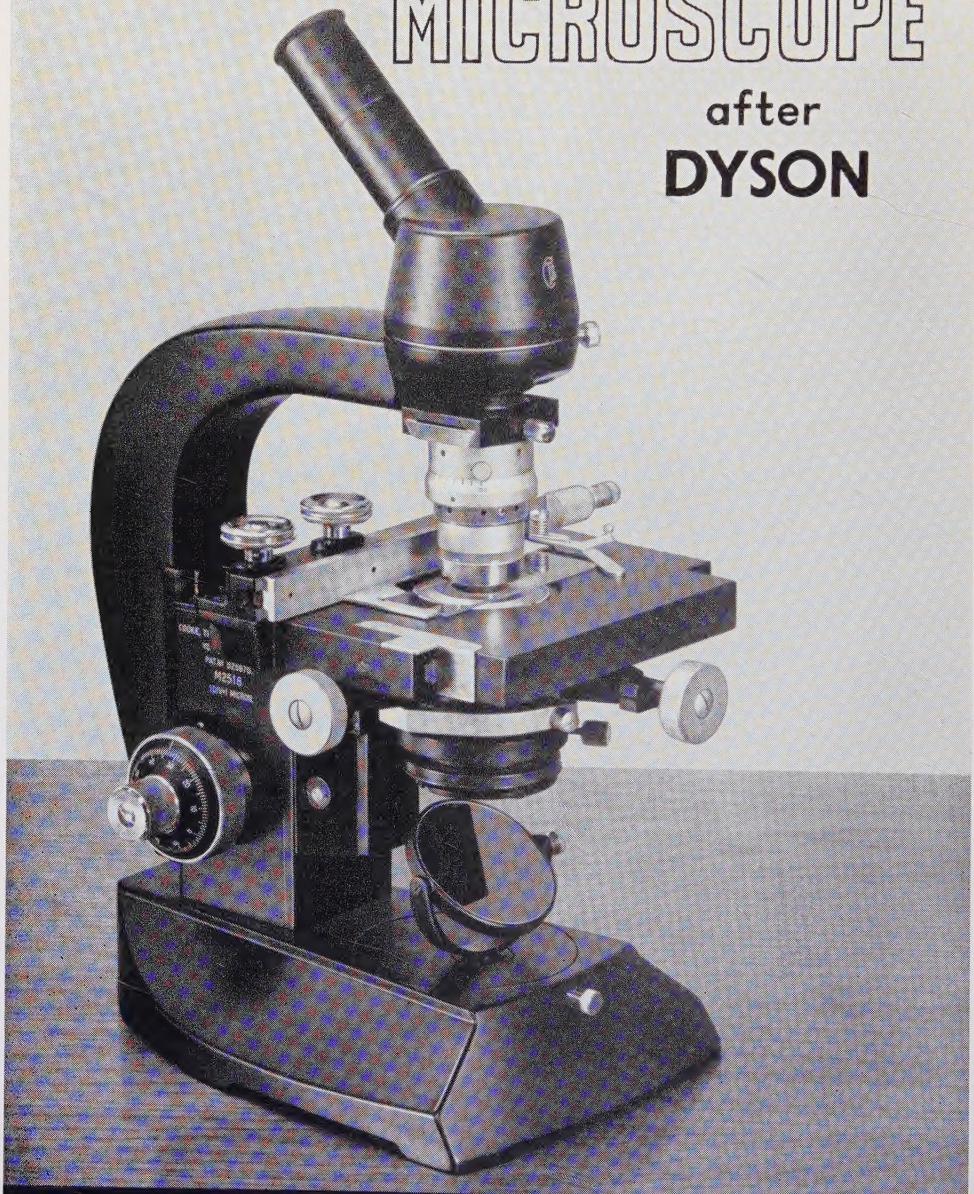

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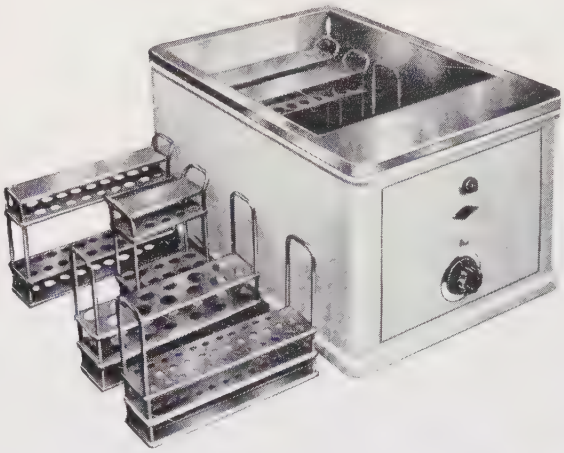

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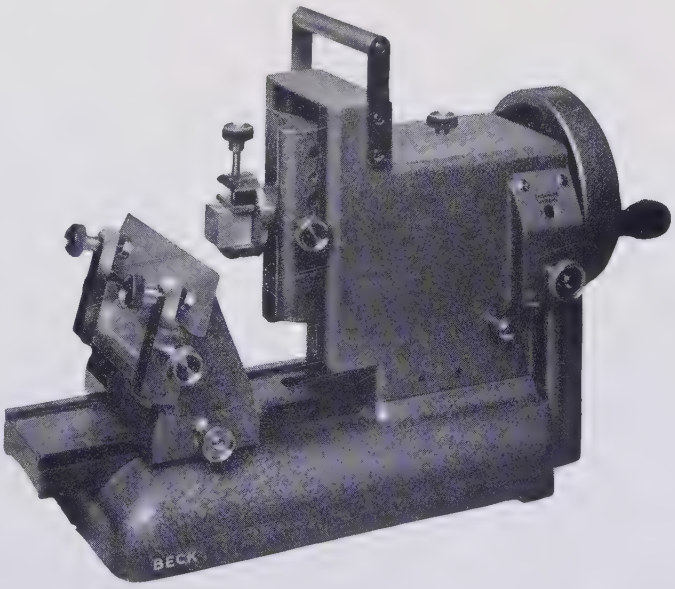

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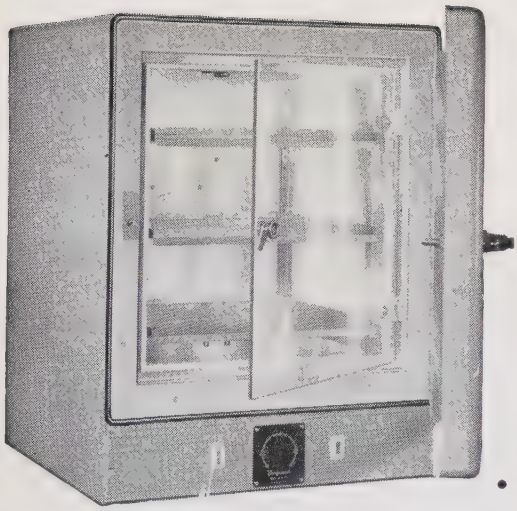

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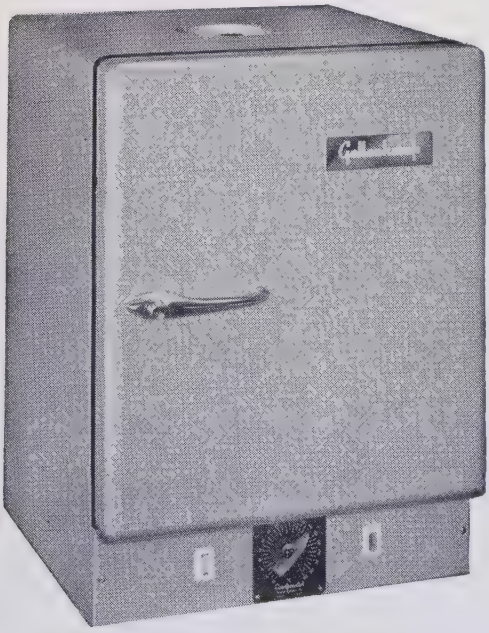

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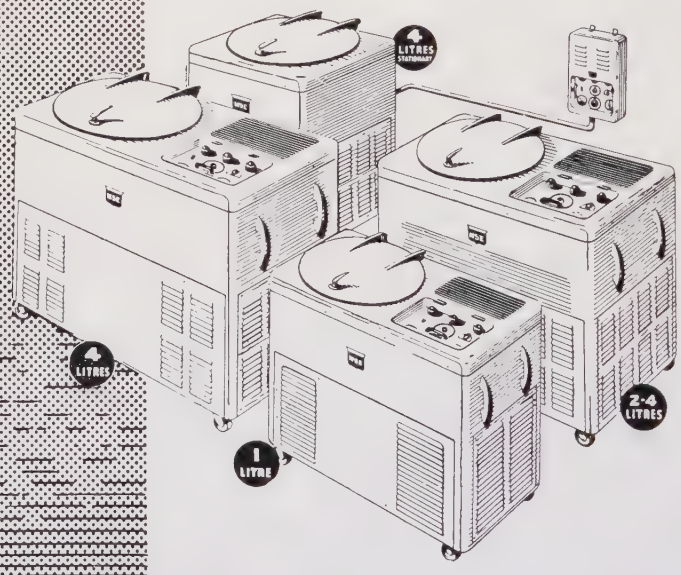

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CONTENTS OF THE APRIL ISSUE

General Meeting
Aluminium for the Dairy Industry, by J. C. BAILEY, B.Sc., F.I.M.
Stainless Steels: Their Uses and Maintenance in the Dairy, by G. H. BOTHAM and the late J. R. CUTTELL
Plastics in the Dairy Industry, by A. WALKER, B.Sc., A.R.I.C.
Correspondence
A Comparison of the Efficiency of a Hypochlorite and Quaternary Ammonium Disinfectant in Udder Wash Waters, by D. A. MCKENZIE and ANNE ROSBOROUGH.
Personalia.
Tank Chlorination of Upland Farm Water Supplies, by D. L. BOWDEN, L. F. BATTLE and S. B. THOMAS.
Problems in Connection with Condensing of Milk, by K. V. V. SANDERSON.
Section Notes.
An Investigation of the Effect of Ultra-High-Temperature Treatment on the Freezing-Point and Composition of Milk, by R. ASCHAFFENBURG, H. BURTON, S. J. ROWLAND and C. C. THIEL.
The Butterfat Testing Scheme as Introduced by the Milk Marketing Board and the Distributors of Milk on 1st October, 1957, by G. R. ROWBOTHAM.
Book Reviews.

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A POLARLY FLAGELLATED MEMBER OF THE ENTEROBACTERIACEAE

By KATHLEEN A. COOK

University College of Wales, Aberystwyth

SUMMARY: An organism which has been frequently isolated from the rhizosphere of plants of *Lolium perenne* (Perennial Rye-grass) is described; it has the typical morphology and flagellation of *Pseudomonas* but the biochemical reactions of *Klebsiella*.

It is possible that some motile members of the Enterobacteriaceae may have a flagella arrangement different from that which is accepted as typical, but that too few strains have been examined for this to be realized. It is of interest therefore to record the characteristics of an organism isolated from the rhizosphere of *Lolium perenne* (Perennial Rye-grass) in experimental plots at the Institute of Rural Science, University College of Wales, Aberystwyth, because it is a typical member of the Enterobacteriaceae, except that it has polar instead of peritrichous flagella. The morphology, cultural and physiological characters of the organism have been studied. Standard bacteriological methods have been used except where stated.

Description

Morphology. The organism is a motile rod 1·0–3·0 μ long by 0·5 μ broad, with parallel sides and rounded ends. Some cocco-bacillary forms with convex sides are usually present, and some larger forms with a constriction in the middle. The cells are arranged singly, with a few end-to-end pairs. The predominant form of flagellation is a single polar flagellum, shown in Plate 1 in a preparation treated with Leifson's flagella stain (Leifson, 1951). However, a few cells have a tuft of polar flagella and some (especially those with a constriction in the middle) have flagella at both ends. Cells from a 48 hr culture on nutrient agar are Gram-negative and stain evenly.

Cultural characteristics. Colonies grown on nutrient agar for 48 hr at 22° are smooth and round (0·5–1·0 mm in diameter), with entire edges. They have no pigment, and are semi-translucent. They are easily emulsified and have a butyrous consistency. Growth on nutrient agar slants is abundant, smooth, colourless and semi-translucent. In nutrient broth growth is uniformly turbid, with a slight deposit. The optimum temperature of growth is 20–25° with poor or no growth at 37°, and the organism is a facultative anaerobe.

Physiological characteristics. Acid and gas are formed from glucose, sucrose, lactose, maltose and mannitol. Acid and gas are formed in lactose-bile salt broth incubated at 22° and 30°, but not at 37° or 44°. In litmus milk acid is produced in 2 to 3 days at 22°, and curdling occurs after prolonged incubation. Indole is not

produced. The methyl red reaction is negative, and acetylmethylcarbinol is formed in glucose phosphate broth. Citrate is utilized. Gelatin is not liquefied (after 21 days at 22°). Urea is hydrolyzed. Nitrates are reduced to nitrites. Tributyrin is not hydrolyzed. Catalase is produced, but not oxidase.

Type strain. A culture has been deposited in the National Collection of Type Cultures, London.

Discussion

If the arrangement of flagella were used as the basis of classification of the species described above, it might well be regarded as a *Pseudomonas*, as motile members of this genus have polar flagellation. But it should not be placed in this genus, because of its metabolic activity, particularly the ability to produce acid and gas from various sugars including lactose. In this it resembles a member of the Enterobacteriaceae; but bacterial species in this family, if motile, have peritrichous flagella (Breed, Murray & Hitchens, 1948).

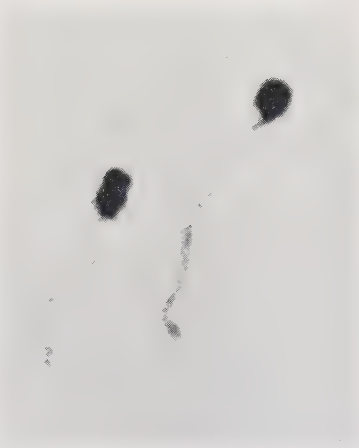
If it is to be included in the Enterobacteriaceae, how should it be classified? According to the Report of the Coli-Aerogenes Sub-Committee of the Society for Applied Bacteriology (Report, 1956), it should be classed as *Klebsiella aerogenes* I (on biochemical grounds only, as the report does not mention motility or flagellation). But, under Kauffmann's classification (1954) the organism cannot be regarded as a *Klebsiella pneumoniae* if it is motile; but it could be a non-gelatin-liquefying strain of *Cloaca cloacae*. Edwards & Fife (1955) have reported strains of the Klebsiella-Aerogenes group of bacteria which were motile, but did not liquefy gelatin, and considered that these strains were atypical *cloacae*. Cowan (1957) also considered that motile strains should not be included in *Klebsiella*, so that it is better to regard the species described in this paper as a polarly flagellated strain of *Cloaca cloacae*. However, this paper is not concerned with the niceties of taxonomy, but with the report that organisms with the biochemical characteristics of *Klebsiella* can have polar flagella, which have always been considered typical, not of that genus, but of *Pseudomonas*.

In 1936 Kluyver & van Niel suggested a new genus, *Aeromonas*, to include rod-shaped bacteria with polar flagella which fermented sugars in a similar way to *Aerobacter* (*Klebsiella*). Asai, Okumura & Tsunoda (1956) described a new genus *Kluyvera*, which consists of polarly flagellated rods with the biochemical reactions of *Escherichia* or *Citrobacter*. It is possible that a series of polarly flagellated species exists, whose metabolism runs parallel with that of the Enterobacteriaceae; if this is so, then the species described in this paper would be another member of this series.

The assistance of Mr. E. Griffiths (Department of Agricultural Botany, University College of Wales, Aberystwyth) in the photographic work is gratefully acknowledged. I am indebted to Mr. S. B. Thomas (N.A.A.S., Trawscoed, Aberystwyth) for his help in the preparation of this report.

References

Asai, T., Okumura, S. & Tsunoda, T. (1956). On a new genus, *Kluyvera*. *Proc. imp. Acad. Japan*, **23**, 488.



KATHLEEN A. COOK — A POLARLY FLAGELLATED MEMBER OF THE ENTEROBACTERIACEAE.
PLATE 1

Breed, R. S., Murray, E. G. D. & Hitchens, A. P. (1948). *Bergey's Manual of Determinative Bacteriology*, 6th ed. London: Baillière, Tindall & Cox.

Cowan, S. T. (1957). *Pers. comm.*

Edwards, P. R. & Fife, M. A. (1955). Studies on the Klebsiella-Aerobacter group of bacteria. *J. Bact.* **70**, 382.

Kauffmann, F. (1954). *Enterobacteriaceae*, 2nd ed. Copenhagen: Ejnar Munksgaard.

Kluyver, A. J. & van Niel, C. B. (1936). Prospects for a natural system of classification of bacteria. *Zbl. Bakt.* (2 *Abt.*) **94**, 369.

Leifson, E. (1951). Staining, shape, and arrangement of bacterial flagella. *J. Bact.* **62**, 377.

Report (1956). The nomenclature of coli-aerogenes bacteria. *J. appl. Bact.* **19**, 108.

Explanation of Plate

Photomicrograph of cells showing polar flagella. Leifson's flagella stain. Magnification ×3300.

(*Received* 8 *August*, 1957)

Baker, L. E. A. & Billing, E. (1958). *J. appl. Bact.* **21** (1), 4–10.

FURTHER OBSERVATIONS ON THE BACTERIAL CONTENT OF WATER IN WATERCRESS BEDS

By LILY A. E. BAKER and EVE BILLING

National Agricultural Advisory Service, Wye

SUMMARY: Observations on two watercress beds in Kent have confirmed previous findings (Jones & Baker, 1955) that even in comparatively well-protected beds the coli-aerogenes content of the water may be high in summer. They have also shown that in spite of circumstances which would lead one to expect pollution, as in the case of one bed, the coli-aerogenes content can be low in winter. The season appears to influence the content in the water of coli-aerogenes bacteria, pectate liquefying bacteria and those capable of growing at 37°, but in the case of those growing at 22° other factors can play a more important part, as higher counts were not uncommon in winter. Seasonal effects were less marked with inlet samples; they were less influenced by atmospheric temperature, and little extraneous contamination occurred. Numbers increased as the sampling point moved from inlet to outlet.

Observations on the bacterial content of water in 16 watercress beds were made by Jones & Baker (1955), but only a few samples were studied from each bed. Walters, Cousins, Bailey & Burton (1954) investigated two watercress beds which were known to be polluted, and described a method of disinfecting watercress using hypochlorite. A further report on the successful use of hypochlorite has been made recently by Walters, Driver & Bailey (1957).

To obtain more detailed information on the fluctuations in the bacterial quality of the water, two watercress beds were selected, and frequent samples were taken over a period of one year.

Materials and Methods

Watercress beds. Two beds of rather different type were selected, one (A) small and natural, the other (B) large and well cultivated. Both were situated in rural areas in Kent.

The small bed A was fed by several springs breaking from the chalk. The flow of water was never rapid, and in summer became quite sluggish as some of the springs dried up. Owing to the inadequacy of the fencing, which did not enclose the source and the top of the bed, stock sometimes had access to the water and consequently contaminated the main flow. The spring used for the first sampling point flowed out at the base of a steep grass bank, and was well protected from contamination by its position.

Bed B was much more extensive than bed A and was divided up by concrete partitions into a number of small areas. Several powerful springs supplied a plentiful flow of water which was well regulated, and showed no diminution in quantity even at the end of a hot dry summer. The surroundings of the bed were cultivated chiefly for fruit growing. No stock were observed anywhere near the bed.

Neither bed had side drainage channels, so both must have received some surface drainage. Plenty of animal and vegetable life, protozoa, shrimps, flatworms, water-fleas, algae and diatoms, was observed in the water. Wherever the growth of the

cress was poor, duckweed and algae multiplied vigorously. In early summer, the vegetation on the banks grew tall. It was scythed back about August at bed B, when cleaning out and replanting was systematically carried out. Partial re-planting was carried out at bed A in July, but the vegetation on the banks was left to die back naturally. Picking and marketing of the cress took place from October to April, though not a great deal was harvested from bed A.

Sampling. Water samples were taken from June 1955 to June 1956. At least two sets were taken from each bed each month, with the exception of December, when only one set was taken. The sampling points were: the inlet, a point in the middle of the bed (two points in bed B), and the outlet. In all, 200 samples were taken, 87 from bed A, and 113 from bed B.

Testing. Samples were examined within 4 hr of sampling, according to the techniques described in Report no. 71 (Ministry of Health, 1939). The following examinations were made: coli-aerogenes bacteria, MPN/100 ml in 5 days at 30°, 2 days at 37° and 2 days at 44°; colony count/ml, on nutrient agar incubated for 3 days at 22° and 3 days at 37°; pectolytic colonies/ml, on a modification of the pectate agar medium described by Luckhurst (1953), with incubation for 3 days at 22°.

The temperature of the water was recorded at the time of sampling and a pH reading was made on all samples. Records were also kept of the mean atmospheric shade temperature at 9 a.m. on the day of sampling, and the amount of rain which fell in the week before the day of sampling.

Results

Presumptive coli-aerogenes content

Incubation at 37° *and* 44°. The results obtained at these two temperatures were similar. Only the 44° results are therefore given in Table 1. The numbers of *Escherichia coli* were lowest in the inlets, increased at the mid-bed sampling points, and were highest at the outlets. In bed A, the percentages of samples taken in summer with over 100 organisms/100 ml were: none at the inlet, 24% at mid-bed, and 47% at the outlet. All percentages, in this and the other Tables, are given to the nearest whole number.

Table 1. *The incidence of different ranges of presumptive* Escherichia coli *content at the different sampling points*

Range of *E. coli* count (/100 ml)	Percentages of samples from:													
	Bed A						Bed B							
	Inlet		Mid-bed		Outlet		Inlet		Mid-bed 1		Mid-bed 2		Outlet	
	S*	W*	S	W	S	W	S	W	S	W	S	W	S	W
10 or less	94	100	12	67	6	50	71	100	31	100	31	83	12	92
11–100	6	0	65	33	47	33	29	0	56	0	63	8	59	8
101–1000	0	0	18	0	41	17	0	0	13	0	6	8	29	0
Over 1000	0	0	6	0	6	0	0	0	0	0	0	0	0	0
No. of samples	17	12	17	12	17	12	17	11	16	12	16	12	17	12

* S, May–October; W, November–April.

In winter there was little difference between the two inlets, but in summer, bed B had 29% of samples with *E. coli* in excess of 10/100 ml, compared with 6% in bed A (Table 1). In samples taken below the source, bed A showed a higher incidence than bed B.

All samples showed a seasonal trend, with higher numbers in summer. This is well illustrated by the outlet samples from bed B (Fig. 1). The variation was less marked in the inlet samples, and also in those taken from mid-bed sampling point 1, in bed B, a point relatively near the source. The other mid-bed samples showed a similar pattern to the outlets.

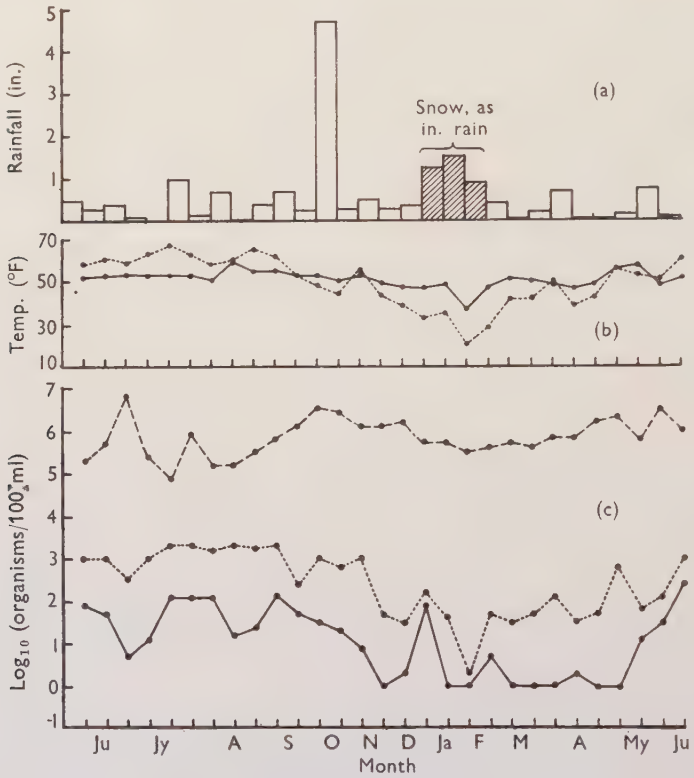


Fig. 1. Rainfall (a), air and water temperatures (b) and bacterial content data (c), relating to samples from outlet of watercress bed B. Temperatures: dotted line, atmospheric; continuous line, water. Bacterial content: dashes, colony count at 22°; dots, coli-aerogenes at 30°; continuous line, *E. coli* at 44°.

Incubation at 30°. The incidence of coli-aerogenes organisms at 30° was higher than at 37° and 44° in all samples (Table 2 and Fig. 1), but the seasonal trend and distribution between sampling points and beds were similar.

Incubation for 5 days increased the number of positive tubes, but did not alter the general pattern of results, so only the figures for 3 days incubation are given in Table 2. It was thought that the late development of gas might possibly be due to

anaerobic bacteria. However, when 42 of these delayed positives were streaked on MacConkey's agar, and the growth obtained inoculated back into MacConkey's broth, 39 produced acid and gas in the normal way.

Table 2. *The incidence of different ranges of presumptive coli-aerogenes content (3 days at 30°) at the different sampling points*

Range of presumptive coli-aerogenes count (/100 ml)	Percentages of samples from:													
	Bed A						Bed B							
	Inlet		Mid-bed		Outlet		Inlet		Mid-bed 1		Mid-bed 2		Outlet	
	S*	W*	S	W	S	W	S	W	S	W	S	W	S	W
10 or less	12	50	6	0	0	0	12	73	0	18	0	8	0	8
11–100	35	33	6	33	0	25	59	27	25	64	27	75	6	67
101–1000	29	17	29	58	24	50	29	0	56	18	40	17	59	25
Over 1000	24	0	59	8	76	25	0	0	19	0	33	0	35	0
No. of samples	17	12	17	12	17	12	17	11	16	11	15	12	17	12

* S, May–October; W, November–April.

Colony counts at 37°

Seasonal variation of the colony count at 37° (Table 3) was less marked than that shown by the coli-aerogenes content. At inlet B there was little difference between samples taken in summer and winter, but at all the other sampling points, colony counts in the higher ranges occurred more frequently in summer.

The percentages of samples with counts in the higher ranges increased from inlets to outlets. Inlet B gave lower counts than inlet A. At the other sampling points, little difference was observed between the beds.

Table 3. *The incidence of different ranges of colony count at 37° on standard nutrient agar, at the different sampling points*

Range of colony count (/ ml)	Percentages of samples from:													
	Bed A						Bed B							
	Inlet		Mid-bed		Outlet		Inlet		Mid-bed 1		Mid-bed 2		Outlet	
	S*	W*	S	W	S	W	S	W	S	W	S	W	S	W
30 or less	35	75	18	58	12	17	88	91	25	58	19	67	12	50
31–300	59	25	65	42	59	75	12	9	69	42	69	33	47	50
301–3000	6	0	18	0	29	8	0	0	6	0	12	0	35	0
Over 3000	0	0	0	0	0	0	0	0	0	0	0	0	6	0
No. of samples	17	12	17	12	17	12	17	11	16	12	16	12	17	12

* S, May–October; W, November–April.

Colony counts at 22°

The colony counts at 22° (Table 4) did not show the same seasonal trend as the coli-aerogenes organisms and the 37° colony counts. The inlet samples and those from mid-bed A did show a slight trend towards higher ranges of count in summer, but seasonal variation was not demonstrated among samples taken at the outlet

of Bed A. The mid-bed and particularly the outlet samples from B had a high percentage of samples in the higher ranges in winter (Table 4). Fig. 1 shows peak counts in June and October, but between these peaks, in the period July-August, some of the lowest counts occurred.

Table 4. *The incidence of different ranges of colony count at 22° on standard nutrient agar, at the different sampling points*

Range of colony count (/ml)	Percentages of samples from:													
	Bed A						Bed B							
	Inlet		Mid-bed		Outlet		Inlet		Mid-bed 1		Mid-bed 2		Outlet	
	S*	W*	S	W	S	W	S	W	S	W	S	W	S	W
30 or less	0	17	0	0	0	0	12	36	6	0	0	0	0	0
31–300	41	58	6	17	0	0	76	64	13	0	6	0	0	0
301–3000	41	25	41	75	35	33	12	0	31	25	13	8	29	0
3000–30,000	18	0	35	8	59	67	0	0	50	75	75	92	53	100
Over 30,000	0	0	18	0	6	0	0	0	0	0	6	0	18	0
No. of samples	17	12	17	12	17	12	17	11	16	12	16	12	17	12

* S, May–October; W, November–April.

Numbers increased as the sampling point moved down the bed from the inlet to outlet. Inlet A had a greater percentage of samples in the higher ranges of count than inlet B, but in samples taken below the source the position was reversed, and bed B had the greater percentage of samples in the higher ranges. It is of interest that in the bed where the coli-aerogenes content was least in samples below the source, the incidence of the higher ranges of colony count at 22° was greatest.

Pectolytic organisms

Pectolytic organisms, able to form depressions on pectate agar, occurred in samples from all points, though the numbers were greater in samples taken below the source (Table 5). The colony counts of these organisms were higher than the nutrient agar colony counts at 37°, but lower than the colony counts at 22°. A seasonal trend occurred, with higher numbers in summer.

Table 5. *The incidence of pectolytic organisms at the different sampling points*

Range of pectolytic colony nos. (/ml)	Percentages of samples from:													
	Bed A						Bed B							
	Inlet		Mid-bed		Outlet		Inlet		Mid-bed 1		Mid-bed 2		Outlet	
	S*	W*	S	W	S	W	S	W	S	W	S	W	S	W
30 or less	47	75	0	0	0	0	59	73	13	0	0	0	0	8
31–300	35	25	12	50	6	0	41	27	25	17	0	25	0	17
301–3000	12	0	65	50	59	83	0	0	50	83	44	42	29	58
3001–30,000	6	0	24	0	35	17	0	0	13	0	50	33	65	17
Over 30,000	0	0	0	0	0	0	0	0	0	0	6	0	6	0
No. of samples	17	12	17	12	17	12	17	11	16	12	16	12	17	12

* S, May–October; W, November–April.

pH, temperature and rainfall

The range of pH was not wide (7·1–7·6) and was similar in both beds. There was no seasonal trend. Throughout the year, and in both beds, there was a small rise in pH between source and outlet.

Water temperatures at the inlets were fairly constant throughout the year. In both beds the maximum temperature recorded was 54°F, and the minimum 49°F. The mean for all inlet samples was 50°F.

Samples of water below the source showed greater fluctuations (Fig. 1). The maximum water temperature recorded was 64°F, the minimum 38°F. Mean atmospheric shade temperature varied between a maximum of 67·5°F in July and a minimum of 22·5°F in February.

The highest weekly rainfall recorded was 4·7 inches in October. This was exceptionally high (Fig. 1). From mid-January to the end of February there was a period of continuous and heavy snow.

Discussion

The water supplying the two beds was of good bacteriological quality at the source. In summer, source B had higher numbers of coli-aerogenes bacteria than source A, but the general pattern over the year showed very little difference between them. This confirms the observations of Jones & Baker (1955), when no marked seasonal trend was found in the inlet samples. By the time the water had reached the mid-bed, a considerable population had accumulated; this accumulation continued rather less rapidly from mid-bed to outlet. Surface drainage as well as contamination by birds and small animals would contribute organisms but multiplication probably took place, either in the water or on the cress and other vegetation. A slowing down in the rate of increase in numbers between mid-bed and outlet might be accounted for by competition between different organisms for available food supplies. Gray (1951), in his studies of a Cambridge chalk stream, suggested that this might happen.

Below the inlets, numbers of coli-aerogenes bacteria increased considerably. The seasonal trend was similar to that described by Cuthbert (1954) for farm water supplies. His opinion was that the variation was governed by a combination of rainfall, soil temperature and grazing season, but that no single factor was entirely responsible. Bed A was frequently contaminated by stock drinking near the source, and stock were nearly always grazing in the vicinity of the bed. These conditions did appear to be reflected in the higher incidence of coli-aerogenes bacteria in bed A, compared with B where sources of contamination were more restricted.

Periods of heavy rain could not be directly associated with peaks on the coli-aerogenes curve. Gray (1951) found that although heavy rain raised bacterial numbers in the Cambridge stream the effect was only temporary.

Other factors which may be of importance in determining the incidence of coli-aerogenes bacteria in the two beds were the volume and the rate of flow of water. Bed B had a vigorous and constant flow of water throughout the year, and was much more extensive in area than A. In A, the flow became very sluggish in summer, and the higher coli-aerogenes content might be as much associated with the smaller

and less free-flowing body of water, as with the greater possibilities of contamination.

As contamination with coli-aerogenes bacteria appears to be inevitable, disinfection of packed watercress (Walters *et al.* 1957) would seem to be a valuable routine measure.

The 37° colony counts followed a pattern similar to the coli-aerogenes bacteria, though the seasonal trend was much less striking. The 22° colony counts, however, showed a different trend, and the seasonal pattern was also different. A large number of samples fell in the higher ranges of count in the winter, and though there were some peak counts in June and October, the period of lowest counts was in July-August. With regard to the seasonal trend, two factors which might contribute to the lower numbers in summer were that the cress grew tall and out of the water at that season and that July-August was the time when cleaning and re-planting were carried out; these operations were done much more thoroughly at B than at A. Such low 22° colony counts in summer may not be typical, as Jones & Baker (1955) found that the counts from a number of beds were lower in winter. If the organisms responsible for the 22° colony counts originated from the surface of the cress, bed B, being larger and better cultivated, would be expected to contain greater numbers of these types than A, where the cress growth was thin and patchy.

The comparatively high incidence of pectate liquefying bacteria observed by Jones & Baker (1955) was confirmed. These organisms followed the seasonal trend shown by the coli-aerogenes bacteria rather than that of the 22° colony count.

We should like to thank the watercress growers who allowed us sampling facilities.

References

Cuthbert, W. A. (1954). The suitability of farm water supplies for dairy purposes. *J. appl. Bact.* **17**, 76.

Gray, E. (1951). The ecology of the bacteria of Hobson's Brook, a Cambridgeshire chalk stream. *J. gen. Microbiol.* **5**, 840.

Jones, G. E. & Baker, L. A. E. (1955). Some observations on the bacterial content of water in watercress beds. *J. appl. Bact.* **18**, 415.

Luckhurst, E. R. (1953). A medium for demonstrating pectate enzyme production by bacteria. *Sci. Tech. Ass. Bull.* **3**, 10.

Ministry of Health (1939). *The Bacteriological Examination of Water Supplies. Rep. publ. Hlth med. Subj.* no. 71. London: H.M.S.O.

Walters, A. H., Cousins, C. M., Bailey, C. A. & Burton, F. H. A. (1954). Bacteriological investigation of polluted watercress beds and disinfection of watercress. *J.R. sanit. Inst.* **74**, 874.

Walters, A. H., Driver, B. M. & Bailey, C. A. (1957). Bacteriological investigation of hypochlorite disinfection of packed watercress. *Med. Offr.* **48**, 277.

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Brown, A. D. & Weidemann, J. F. (1958). *J. appl. Bact.* **21** (1), 11–17.

THE TAXONOMY OF THE PSYCHROPHILIC MEAT-SPOILAGE BACTERIA: A REASSESSMENT

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SUMMARY: A study was made of selected properties of 189 psychrophilic bacteria isolated from chilled beef and associated sources. Of these, 182 were Gram-negative and 7 were Gram-positive. Of the former, 128 were typical pseudomonads with polar flagella. They were resistant to penicillin and oxidized glucose to acid. There were 52 non-motile Gram-negative bacteria of which 42 were judged to be pseudomonads on the basis of penicillin resistance and the oxidation of glucose to acid. Flagellar characters were associated with the production of green pigment, with the production of acid from lactose and with resistance to several antibiotics.

The identity of the bacteria associated with the spoilage of fresh meat at low temperature has long been a matter of some doubt. Haines (1933) stated that the genus *Achromobacter* was predominant among these organisms, while earlier work in this laboratory (Empey & Scott, 1939) indicated that on Australian beef, the genus *Achromobacter* may represent 90% of the total bacterial population capable of growth below 0°.

More recent reports have indicated the greater importance of the genus *Pseudomonas*, especially the non-pigmented strains (Ayres, Ogilvy & Stewart, 1950; Kirsch, Berry, Baldwin & Foster, 1952; Ayres, 1955). That this change in emphasis is due in part to changed criteria introduced in the 6th edition of Bergey's Manual (Breed, Murray & Hitchens, 1948) has been noted by Ayres (1955). Even in this edition the family Achromobacteriaceae is not well defined and the one positive criterion which may be used to distinguish this family from the Pseudomonadaceae is the type of flagellation of motile cells. Distinctions of this kind were not made by Haines (1933), who described 8 typical 'Achromobacteria', 4 of which were non-motile and the other 4 monotrichous. Similarly, the distinction was not made by Empey & Scott (1939), who allocated organisms to the genus *Pseudomonas* only if they produced water-soluble green pigment (Scott, personal communication).

The availability of a collection of cultures derived from the original isolations of Empey & Scott made it convenient to reconsider the identity of these strains, together with a somewhat smaller number of cultures recently isolated from beef held at low temperatures.

Materials and Methods

Organisms

A total of 189 organisms was selected for study. Of these 129 were from the collection maintained on nutrient agar (0·3% meat extract, 0·5% peptone) at about 0° for the

last two decades with transplants every 6–12 months. After each transplant they were incubated at 20° for 2–3 days before being placed in storage. These 129 cultures included 66 formerly listed as *Pseudomonas*, 56 formerly listed as *Achromobacter* and 7 unclassified. The remaining 60 cultures were isolated in this laboratory from beef stored at 0°. Four of these were isolated in 1953 and the rest in 1956.

Diagnostic criteria

All organisms were examined in respect of the following properties, the appropriate media being inoculated by loop from 2–3 day cultures in nutrient broth (0·3% meat extract, 0·5% peptone). For all purposes except temperature relations the cultures were incubated at 20°.

Temperature relations. These were recorded as the ability to grow overnight in nutrient broth at 37° and the number of days to definite turbidity in nutrient broth at 0°.

General colonial and cellular morphology. Colonies were examined after growth on nutrient agar for 2–3 days. Cellular morphology was studied in Gram-stained preparations from overnight cultures in peptone water and by phase-contrast in water mounts from 2-day cultures on nutrient agar.

Motility and number, type and position of flagella. Observations on motility were made on overnight cultures in peptone water. Where no motility was observed further observations were made on overnight cultures in nutrient broth and a number of representative non-motile cultures was stained for flagella by the method of Leifson (1951) after growth for 18–24 hr on nutrient agar plates.

Production of water soluble green pigment. Cultures were incubated for 14 days at 20° in the asparagine medium of Georgia & Poe (1931). For positive tests the number of days' incubation necessary to produce a band of pigment approximately 3 mm deep was noted.

Production of acid from glucose or lactose. The distinction between oxidative and fermentative production of acid from glucose was made by the paraffin layer technique of Hugh & Leifson (1953). Production of acid was assessed with bromo-cresol purple.

Sensitivity to various antibiotics. This test was included because of the work of Shewan, Hodgkiss & Liston (1954). Patterns of sensitivity were determined using standard antibiotic tablets and nutrient agar plates incubated for 1 or 2 days. The following antibiotic tablets (Evans Medical Supplies Ltd.) were used; penicillin (2·5 international units), streptomycin (80 μg), chloramphenicol (100 μg), Aureomycin (10 and 100 μg), Terramycin (10 μg), erythromycin (10 μg), tetracycline (100 μg). Various other tests were applied in special cases.

Results

The Gram-negative organisms

All the Gram-negative organisms were rods with round or slightly rounded ends, occurring singly and in pairs and occasionally in chains. Average dimensions after growth overnight in peptone water were in the order of 0·5–1 × 1·5–3μ, but it was not uncommon to observe within cultures a range of cell lengths from coccobacillary

to elongated rods and sometimes filaments. When filaments occurred branching was never observed. Colonies tended to be smooth, round and entire and, with one exception, colourless. The diameter of colonies after 2 days on nutrient agar usually fell within the range 0·5–3 mm.

Motile organisms. The results of observations on the number and position of flagella are given in Table 1, according to source. It will be seen that 128 organisms had one polar flagellum or more, while Table 2 shows that all these cultures not only produced acid from glucose under aerobic, though not under anaerobic, conditions but also were resistant to penicillin. The latter character has been claimed

Table 1. *The distribution of* 182 *Gram-negative strains into flagellar types*

Flagellar type	Stock cultures			Recent isolates	Totals
	Listed as *Pseudomonas*	Listed as *Achromobacter*	Unclassified		
Non-motile	4	29	6	13	52
Polar, monotrichous	22	22	0	37	81
Polar, multi- and lophotrichous*	40	1	0	6	47
Peritrichous	0	1	0	0	1
Sub-polar	0	1	0	0	1
Totals	66†	54†	6	56	182

* The distinction between these two terms is that of Leifson (1951). Six lophotrichous strains were observed among the stock cultures. The rest were multitrichous.

† The ratio of '*Pseudomonas*' to '*Achromobacter*' was dictated by the number of surviving stock cultures.

Table 2. *The incidence of selected physiological properties among the Gram-negative strains according to the three principal types of flagellation*

Physiological property	Incidence of selected physiological properties among							
	52 non-motile strains		81 polar monotrichous strains		47 polar multi- & lopho-trichous strains		All strains (180)	
	No.	%	No.	%	No.	%	No.	%
Oxidation of glucose to acid	46	88	81	100	47	100	174	97
Oxidation of lactose to acid*	4	8	42	52	3	6	49	27
Fermentation of glucose to acid	1	2	0	0	0	0	1	1
Growth at 37°	2	4	9	11	12	26	23	13
Sensitivity to penicillin	7	13	0	0	0	0	7	4
Production of green pigment	0	0	15	19	43	91	58	32

* Negative results were recorded after 40 days.

by Shewan *et al.* (1954) to be a property of *Pseudomonas* species in contrast to species of *Achromobacter*, which were found to be sensitive to penicillin. Table 2 also shows that 90% of the multitrichous strains and 18% of the monotrichous strains produced a water soluble green pigment. These 128 cultures are believed all to be members of the genus *Pseudomonas*. The two other motile organisms comprised a coli-aerogenes organism with peritrichous flagella and a possible member of the genus *Aeromonas* (Kluvyer & van Niel, 1936) with up to three sub-polar flagella. Examples of the four types of flagellar arrangement are shown in Fig. 1.

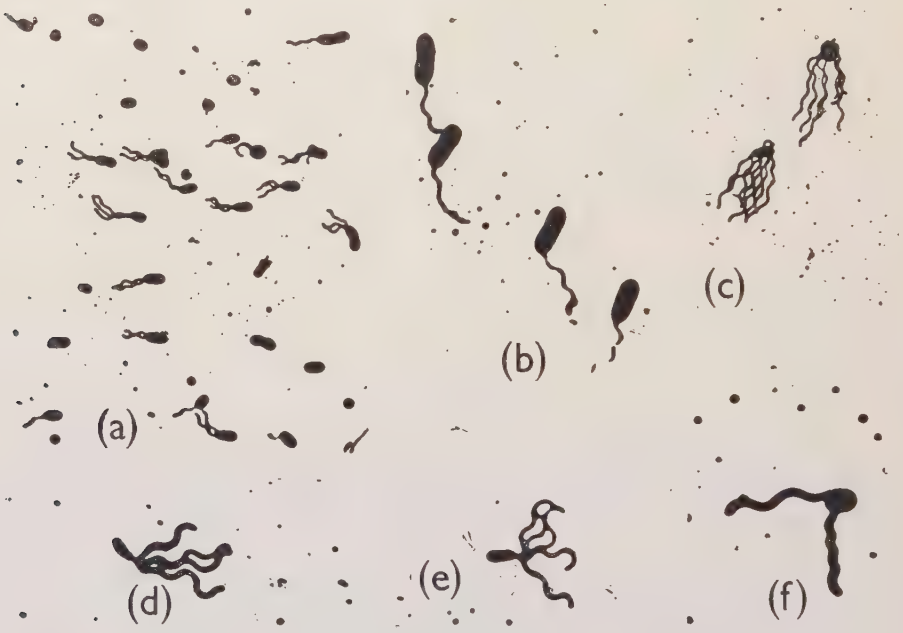

Fig. 1. Examples of the four types of flagellar arrangement listed in Table 1. All preparations were stained with Leifson's flagella stain (1951).

(a) Typical field of a multitrichous organism showing up to 3 flagella at one pole (×1090).

(b) Typical field of a monotrichous organism. The cells were larger than average for most of the monotrichous species (×1100).

(c) The peritrichous organism (×1090).

(d) & (e) Individual multitrichous cells. Flagella were heavily stained for the photographs and under these conditions fewer flagella could be counted than in less heavily stained preparations. Species with up to 14 flagella at one pole were observed (×2450).

(f) The organism with sub-polar flagella (×2450).

Non-motile organisms. The 52 non-motile organisms were distributed among all the sources listed in Table 1. However, the old isolates formerly listed as *Pseudomonas*, which comprised 36% of all the Gram-negative organisms, yielded only 8% of the non-motile organisms. None of the 52 strains produced green pigment under the conditions of testing (Table 2), although 9 of them failed to grow in the asparagine

salts medium used for this test. Of these 52 organisms, 42 were judged to be pseudomonads on the basis of their ability to oxidize glucose to acid and their resistance to penicillin. Of the remaining 10 organisms, 1 produced acid from glucose under anaerobic conditions and the remaining 9 were either sensitive to penicillin or did not produce acid from glucose, or both. Four of these 9 organisms had both these properties and might, therefore, be placed in the Achromobacteriaceae. The other 5 included 3 organisms which oxidized glucose and lactose to acid but which were sensitive to penicillin. These 3 organisms are left unclassified. It is of interest to note that these 9 organisms were the same ones which, as mentioned above, failed to grow in the asparagine-salts medium used for testing pigment production.

Association between flagellation and physiological characters. It is a matter of some interest that an association has been revealed between the number of flagella and certain physiological characters. Table 2 shows that pigment production is associated with the presence of flagella and that none of the non-motile organisms produced green pigment. Similarly the ability to grow at 37° as well as at 0° was more frequent in motile than in non-motile organisms, while the ability to oxidize lactose to acid was observed most frequently in monotrichous strains. A general tendency for sensitivity to several antibiotics to be less frequent among the motile strains, and in particular among the lopho- and multitrichous strains, is revealed in Table 3.

Table 3. *The incidence of sensitivity to antibiotics other than penicillin among variously flagellated groups of probable* Pseudomonas *species*

Antibiotic and amount used (μg)	Incidence of antibiotic sensitivity among					
	42 non-motile strains		81 monotrichous strains		47 lopho- and multitrichous strains	
	No.	%	No.	%	No.	%
Aureomycin, 10	39	93	65*	80*	14	30
Aureomycin, 100	42	100	80	99	45	96
Chloramphenicol, 100	42	100	67	83	30	64
Erythromycin, 10	13	31	10	12	0	0
Streptomycin, 80	42	100	81	100	46	98
Terramycin, 10	40	95	59	73	17	36
Tetracycline, 100	42	100	79	98	42	89

* Represents 28 (64%) of the stock cultures and 37 (100%) of the recent isolates.

The Gram-positive organisms

Seven of the 189 cultures were Gram-positive. Four of these were probably species of *Corynebacterium*. The other three were: a yellow rod with a polar flagellum, a non-pigmented rod and a coccus.

Discussion

The principal conclusion arising from these experiments is that the psychrophilic Gram-negative bacteria growing on beef are almost all pseudomonads. This corrects

an earlier statement from this laboratory that species of *Achromobacter* accounted for 90% of the psychrophilic bacteria on Australian beef (Empey & Scott, 1939). As the present results support the findings of two groups of workers in North America (Ayres *et al.* 1950; Kirsch *et al.* 1952) it is likely that they would apply also in other areas.

Of the 182 Gram-negative organisms studied, 170 (93%) are believed to be species of *Pseudomonas*. The 170 cultures may be divided into three groups for each of which the conclusion has a different degree of certainty.

The first group of 58 organisms all produced green pigment, all had one polar flagellum or more, all produced acid oxidatively from glucose and all were resistant to penicillin. These are certainly species of *Pseudomonas*. The next group of 70 organisms did not produce a water soluble green pigment, but possessed the other three attributes of the first group, and almost certainly comprises species of the same genus and certainly within the same family. The 42 organisms of the third group produced neither pigment nor flagella and their identity is, therefore, much less definite. It is suggested, however, that they also be regarded as species of *Pseudomonas* which lack the ability to produce flagella and a water-soluble green pigment.

The possibility that non-pigmented or non-motile strains might arise from motile pigmented strains receives some support from the present study. Twenty-one strains, originally classified by Empey & Scott (1939) as *Pseudomonas* spp. on the basis of pigment production, failed to produce pigment in the recent tests. This number includes 3 multitrichous, 14 monotrichous and 4 non-motile bacteria, all of which on the criteria used in this study are members of the genus *Pseudomonas*. As pigment production was strongly associated with multi- and lophotrichous flagellation it is possible that among the stock cultures originally classified as *Pseudomonas* spp. there has been a progressive loss for some strains of capacity to produce pigment and/or flagella during the years they were maintained in culture.

The association between flagellar type and other properties (Tables 2 and 3) suggests that, at least within this group of Gram-negative aerobic polar flagellated organisms (and related non-motile forms), the number of flagella might be more closely related to cellular physiology than has hitherto been supposed. A more intensive examination of this possibility might well prove valuable. Whether or not it is conceded that the non-motile oxidative bacteria of this survey should be regarded as species of *Pseudomonas*, the possession of an oxidative carbohydrate metabolism together with their other listed properties closely relates them to the motile species of that genus. The very high correlation of oxidative carbohydrate metabolism with psychrophilic temperature relations might be of fundamental importance but it is clear that a much wider survey must be conducted before any generalizations can be made to include bacteria from other sources.

Of some use in an approach to understanding the physiology of the psychrophilic meat-spoilage bacteria is the appreciation of the numerical importance of the genus *Pseudomonas*. Some aspects of the physiology and biochemistry of selected species of this genus are now well understood whereas relatively little information of a similar kind is available at the present time about any species of *Achromobacter*.

The authors are indebted to Mr. F. Grau (present address, Department of Agricultural Bacteriology, University of Wisconsin, U.S.A.) for isolating most of the recent species, and to Mr. J. A. Robinson for taking the photographs.

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References

Ayres, J. C. (1955). Microbiological implications in handling, slaughtering and dressing of meat animals. *Advanc. Food Res.* **6**, 109.

Ayres, J. C., Ogilvy, W. S. & Stewart, G. F. (1950). Post mortem changes in stored meats. I. Micro-organisms associated with development of slime on eviscerated cut-up poultry. *Food Tech., Champaign*, **4**, 199.

Breed, R. S., Murray, E. G. D. & Hitchens, A. P. (1948). *Bergey's Manual of Determinative Bacteriology*. 6th ed. Baltimore: Williams & Wilkins.

Empey, W. A. & Scott, W. J. (1939). Investigations on chilled beef. Part I. Microbial contamination acquired in the meatworks. *Bull. Coun. sci. industr. Res. Aust.* no. 126.

Georgia, F. R. & Poe, C. F. (1931). Study of bacterial fluorescence in various media. I. Inorganic substances necessary for bacterial fluorescence. *J. Bact.* **22**, 349.

Haines, R. B. (1933). The bacterial flora developing on stored lean meat, especially with regard to 'slimy' meat. *J. Hyg., Camb.*, **33**, 175.

Hugh, R. & Leifson, E. (1953). The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram-negative bacteria. *J. Bact.* **66**, 24.

Kirsch, R. H., Berry, F. E., Baldwin, C. L. & Foster, E. M. (1952). The bacteriology of refrigerated ground meat. *Food Res.* **17**, 495.

Kluyver, A. J. & van Niel, C. B. (1936). Prospects for a natural system of classification of bacteria. *Zbl. Bakt.* (2 *Abt.*) **94**, 369. (Cited by Leifson & Hugh, 1953.)

Leifson, E. (1951). Staining, shape and arrangement of bacterial flagella. *J. Bact.* **62**, 377.

Leifson, E. & Hugh, R. (1953). Variation in shape and arrangement of bacterial flagella. *J. Bact.* **65**, 263.

Shewan, J. M., Hodgkiss, W. & Liston, J. (1954). A method for the rapid differentiation of certain non-pathogenic, asporogenous bacilli. *Nature, Lond.*, **173**, 208.

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A NEW METHOD OF COLLECTING ALLANTOIC FLUID FROM EMBRYONATED EGGS

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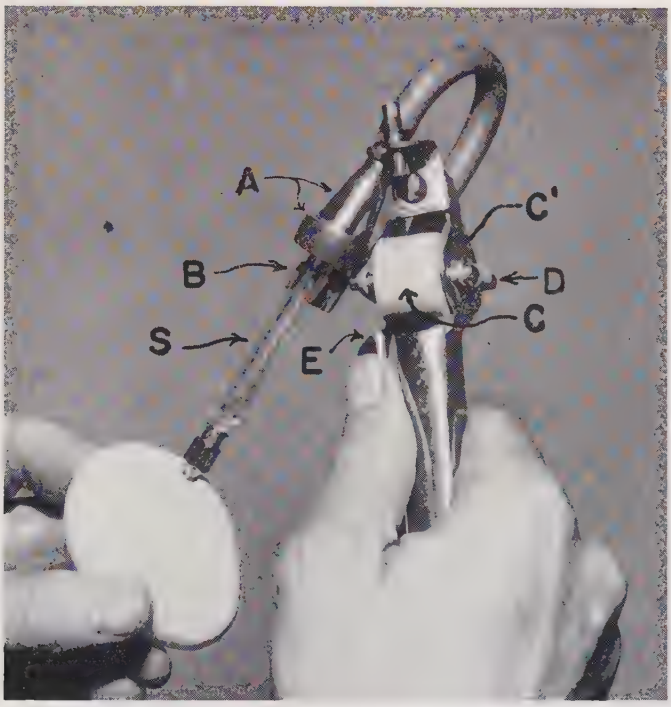
SUMMARY: An apparatus is described for collecting by aspiration allantoic fluid from embryonated eggs infected with influenza or Newcastle disease virus. The essential part of the apparatus is a specially designed 'automatic pipette' permitting good control of the flow of aspirated fluid.

In the production of influenza and Newcastle disease vaccines, the viruses are cultivated in the allantoic cavity of embryonated eggs. After suitable incubation, the allantoic fluid containing the virus is normally removed either by aspiration with a pipette, after opening the shell above the air sac and tearing the membranes, or by a short, perforated hypodermic needle attached to a syringe, the eggs remaining intact except for the small hole permitting the insertion of the needle. The first method has the advantage of permitting visual inspection of the egg contents and the rejection of those obviously contaminated, but it is inferior to the second method on two counts: aspiration by needle is not only quicker, as the step of cutting away the shell and tearing the membranes is omitted, but also it prevents contamination by airborne micro-organisms and eliminates the need for special hoods.

The method advocated here is an improvement of the needle aspiration method, making use of an 'automatic pipette' devised and produced by A. Goldberg, 8, Zhabotinsky Street, Tel-Aviv, Israel (Israel Patent 9008 and foreign patents pending). Some details of the construction and functioning of the 'pipette' are shown in Plate 1. An 18 swg hypodermic needle is cut to a length of $\frac{3}{4}$ in. with the aid of a rotating steel disc, 6–8 grooves are cut into it (alternately on opposite sides) and it is mounted on the barrel of a $\frac{1}{4}$ or $\frac{1}{2}$ ml tuberculin syringe (S, Plate 1a). The barrel, having previously been cut at the non-graduated end and fire-polished, can be inserted into the rubber seat (A) of the pipette and held there by a clip (B). The other end of the seat is connected to rubber tubing which passes, and is pinched off, between two rubber cylinders (C,C′), which are held together by two spiral springs (D), one on each side. Depressing the lever (E) releases the pressure on the tubing and permits air or fluid to pass freely through it. Beyond the cylinders the tubing passes through the hollow handle of the 'pipette' and is connected to glass tubing, which in turn is inserted through a rubber stopper fitting the collection bottle (Plate 1b). Suction is provided through a second glass tube passing through the stopper and connected through a cotton wool filter to a trap and a vacuum source, such as a water pump. Another view of the device for controlling the flow of air or fluid is shown in Plate 1c.



(*a*)

(*b*)

(*c*)

A. Kohn — A new method of collecting allantoic fluid from embryonated eggs.
Plate 1

The automatic pipette, assembled with the rubber tubing, rubber stopper and filter, syringe, and needle (protected by a sterile tube), may be sterilized in an autoclave. When this is done, a wooden or metal wedge should be inserted between the cylinders (C,C′) to keep them apart and so prevent the rubber tubing from being squeezed between them during sterilization. Just before use the wedge is removed and the tension of the springs (D) tested by depressing and releasing the lever (E) several times.

Operation

The water pump is turned on and the safety cock on the trap closed. The handle of the 'pipette' is then grasped in one hand, the needle inserted through the inoculation hole into the egg (Plate 1a), the release lever (E) depressed with the thumb and the suction started. Allantoic fluid moves up the syringe barrel, through the rubber tubing and into the collection bottle. One may stop suction by releasing the pressure on the lever, when the springs pull the rubber cylinders together and the rubber tubing is pinched off, thus stopping the flow. Suction should be stopped when large air bubbles appear in the syringe, indicating that no more allantoic fluid remains in the egg. If, by any chance, blood or yolk is drawn into the syringe, suction is stopped as above, the upper cylinder (C) is rotated by an upward motion of the thumb, which causes the tubing to be squeezed and progressively shortened, so that the unwanted fluid is ejected from the syringe and needle. All this may be done without stopping suction beyond the pinched off section of the tubing. One may also wash the syringe and needle with sterile fluid by rotating the rubber cylinders to and fro with the thumb, while still maintaining the suction ready to operate as soon as the lever is again depressed. When desired, both the needle and the syringe barrel may be replaced by another without dismantling the apparatus.

Where such automatic pipettes are not available, one may use a pinchcock on rubber tubing connected directly to the syringe. With this arrangement, however, when yolk or blood are aspirated, the whole assembly has to be changed, unless inclusion of such materials in the collected allantoic fluid is not considered critical.

The apparatus described here has been successfully used for collecting allantoic fluid from several tens of thousands of eggs. The average time of collection from 100 eggs was 45–50 min and the volume of fluid collected from 12-day old infected embryos was 6·2 ml/egg.

Explanation of Plate

(a) Details of the construction of the automatic pipette (see text).
(b) The pipette connected to the collection bottle.
(c) Another view of the device for controlling the flow of air or fluid.

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THE NUTRITION AND SOME BIOCHEMICAL STUDIES OF *VIBRIO CYCLOSITES* AND *VIBRIO NEOCISTES*

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SUMMARY: *Vibrio cyclosites* and *V. neocistes* grew in a defined medium consisting of a mineral basis, nicotinamide or nicotinic acid and the amino acids methionine, histidine, aspartic acid, phenylalanine and *iso*leucine. Washed suspensions failed to oxidize any of these substances in the Warburg apparatus. It was not possible with these organisms to demonstrate transamination between the five essential amino acids and α-ketoglutaric acid or pyruvic acid.

The organisms *Vibrio cyclosites* and *V. neocistes*, which have the interesting property of being able to decompose aromatic rings, were isolated from soil and described by Gray & Thornton in 1928. *V. cyclosites* can attack phenol and *m*-cresol, and *V. neocistes* attacks naphthalene, and the economic significance of these organisms lies in their ability to destroy soil disinfectants. Ability to decompose aromatic rings is a property also shared by members of the genus *Pseudomonas*, members of which are common contaminants of wounds in man and are also remarkably resistant to antiseptics. Much work has appeared on the nutrition and metabolism of the pseudomonads but little has been done on the two *Vibrio* species. This study forms part of a programme of work on organisms particularly resistant to, or able to metabolize, antiseptics.

Methods

Organisms

V. cyclosites (NCIB 2581) and *V. neocistes* (NCIB 2582) were obtained as freeze-dried cultures from the National Collection of Industrial Bacteria; both were maintained in nutrient broth and on nutrient agar at 22°.

Chemicals and culture media

Unless otherwise stated, all chemicals were of analytical reagent purity. Amino acids and growth factors (referred to hereafter collectively as growth factors) were obtained from Roche Products Ltd.; the antibiotic tablets for inhibition tests were the Sentest tablets of Evans Medical Supplies Ltd.; the vibriostatic compound 0/129, 2:4-diamino-6:7:di-*iso*propylpteridine, was kindly given by Dr. H. O. J. Collier, Pharmacology Dept., Allen & Hanburys Ltd. The nutrient broth contained Lab-Lemco, 1%; peptone, 1%, and sodium chloride, 0·5%; when necessary this broth was solidified with 2% agar. These culture media constituents were Oxoid brand.

The basal mineral medium contained $(NH_4)_2HPO_4.2H_2O$, 0·4%; NaCl, 0·1%; $FeSO_4.7H_2O$, 0·03%; KH_2PO_4, 0·04%, and $MgSO_4.7H_2O$, 0·07%; this was normally prepared at double strength and supplemented as required with various combinations of growth factors and carbon source. Supplemented medium was prepared by aseptic addition of sterilized solutions to previously sterilized basal medium distributed in sterilized test tubes, closed with aluminium caps (Oxo Ltd.). The finished media were heated at 105° for 5 min as a final precaution and incubated at 37° for 3 days and 22° for 5 days as a check on sterility.

Determination of growth requirements

Qualitative. Media were prepared containing various combinations of growth factors, and were inoculated with one 4 mm loopful from a 24 hr culture of the organism in nutrient broth. A particular medium was considered to be capable of supporting growth if normal growth was maintained after not less than 5 serial subcultures using a single loopful from the previous tube as inoculum.

Quantitative. In certain instances the effect of graded doses of a single growth factor was determined. An inoculum was made after serial subculture in minimal medium (see below) into a set of tubes containing graded doses of the factor. From this set a second identical set was inoculated from the tube containing the corresponding amount of factor, and this procedure was repeated until a total of 5 sets had been inoculated. After 48 hr the amount of growth was determined by a total cell count using a Thoma counting chamber, the cells being stained with safranin.

Determination of resistance to antibiotics and vibriostatic compound

Nutrient agar seeded with the organism was poured into 5 in. Petri dishes and allowed to set; the antibiotic tablets were applied directly to the seeded agar, and the vibriostatic compound was added as a saturated aqueous solution in a porcelain antibiotic assay cylinder fixed to the agar by heat.

Oxygen uptake

This was investigated by the direct method of Warburg at 22° and 37° in a gas phase of air. The flasks contained 0·05 M substrate, 0·5 ml; 0·05 M phosphate buffer, pH 7·0 and 7·8, 0·5 ml, or basic mineral medium, 0·5 ml; the side arm contained the cell suspension, 6·0–8·0 mg dry wt/ml, 0·5 ml; the centre well contained 20% (w/v) KOH solution, 0·2 ml and a fluted filter paper 1·2 cm square.

Transamination

The reaction mixture consisted of 0·5 M sodium α-ketoglutarate or sodium pyruvate 0·1 ml; the amino acid, 0·125 M, 0·2 ml; cell suspension, 6·0 mg dry wt/ml, 0·2 ml; 0·05 M phosphate buffer, pH 8·3, 0·1 ml, and water, 0·4 ml. Mixtures were incubated at 22° and 37° for 10, 20 and 60 min with and without the addition of pyridoxamine, 35 μg. Evidence for the formation of glutamic acid or alanine by transamination

was sought by paper chromatography. The reaction was arrested by adding 0·05 ml trichloracetic acid (100% w/v). The reaction mixture, 0·02 ml. and 0·05 ml of 0·125 M glutamic acid or alanine, were placed on No. 4 Whatman paper and chromatographed by the ascending method using butanol-acetic acid as the solvent. The papers were dried, heated at 100° for 15 min, sprayed with 0·1% w/v ninhydrin in *n*-butanol and heated at 80° for 5 min to develop the blue colour.

Results

Both species of organism as obtained from the NCIB occurred as Gram-negative rods, and at no time was the curved shape associated with vibrios noted. Both organisms were without action on litmus milk, starch broth, nutrient gelatin or glucose broth and failed to ferment the sugars, polyhydric alcohols and glycosides normally used in diagnostic bacteriology. Indole was not produced nor were nitrites formed from nitrates. These characteristics agree with those recorded by Gray & Thornton (1928) except that these workers reported that both organisms occurred as curved rods and produced acid from glucose, and that *V. neocistes* liquefied and reddened gelatin.

Difficulty in distinguishing between members of the genera designated in Bergey's Manual of Determinative Bacteriology (Breed, Murray & Hitchens, 1948) as *Vibrio*, *Pseudomonas* and *Achromobacter* is well recognized, and recently Shewan, Hodgkiss & Liston (1954) described a method of differentiation based on the behaviour of these organisms towards certain antibacterial substances. The antagonistic effects of Terramycin (oxytetracycline), penicillin, streptomycin, chloramphenicol and 2:4-diamino-6:7-di-*iso*propylpteridine (O/129) towards *V. cyclosites* and *V. neocistes* were determined and the results, together with those obtained by Shewan *et al.* (1954) are given in Table 1.

Table 1. *The effect of antibacterial compounds on* V. cyclosites *and* V. neocistes *in comparison with observations by Shewan* et al. (1954) *on certain other micro-organisms*

Organism	Antibacterial substances				
	O/129	Strepto-mycin	Penicillin	Chloram-phenicol	Terra-mycin
Pigment-producing pseudomonads *	−	——	−	——	−
Non-pigment-producing pseudomonads *	−	——	−	——	+
Vibrios *	+	——	−	——	+
Pseudomonadaceae *	——	+	−	+ or ++	——
Achromobacteriaceae *	——	++	+	++	——
Bacteriaceae { Gram + ve *	——	−	+	+	——
Bacteriaceae { Gram − ve *	——	+	−	+	——
Vibrio cyclosites	−	++	−	++	+
Vibrio neocistes	−	++	−	++	+

*, data from Shewan *et al.* (1954). −, insensitive; +, antagonized; ——, not tested.

The vibriostatic compound O/129 was not active against *V. cyclosites* and *V. neocistes*, whereas Shewan *et al.* reported it to be active against some 200 strains of vibrios.

Nutritional pattern of the organisms

The effect of the following 24 growth factors was investigated: tyrosine, leucine, alanine, phenylalanine, valine, glycine, cystine, arginine, lysine, asparagine, glutamic acid, aspartic acid, histidine, *iso*leucine, methionine, threonine, tryptophane, aneurine, riboflavine, pyridoxine, pantothenic acid, *p*-aminobenzoic acid, biotin, nicotinic acid and nicotinamide.

A medium was first prepared containing all the factors each at 50 μg/ml together with 1% glucose in the basal medium. The final pH of the medium was 7·4 and good growth, maintained at the same level for at least seven subcultures, was obtained with both organisms. By eliminating growth factors from this medium a minimal nutritional pattern emerged. In the first instance growth was not obtained if nicotinic acid and its amide were omitted from the complete medium; but 0·5 μg/ml of nicotinamide permitted growth which with *V. neocistes* was little less than when the concentration was 100 times as great (Fig. 1). Of the amino acids, methionine, histidine, aspartic acid, phenylalanine and *iso*leucine were found to be essential.

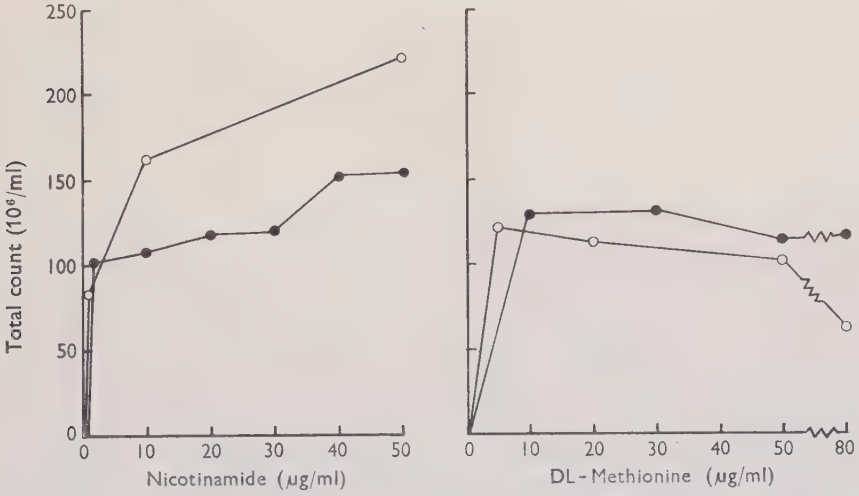

Fig. 1. The quantitative growth responses of *Vibrio cyclosites* and *V. neocistes* to nicotinamide and DL-methionine in a defined medium. Open circles, *V. cyclosites*. Full circles, *V. neocistes*.

As little change in pH was observed during growth, it was possible that the glucose in the medium was not being utilized; this was confirmed when it was found that its omission made no difference. Thus the recipe for a minimal fully defined medium which would support in continuous subculture the growth of both *V. cyclosites* and *V. neocistes* from single 4 mm loop inocula was: nicotinic acid or nicotinamide,

methionine, histidine, aspartic acid, phenylalanine and *iso*leucine in the basic mineral medium.

Methionine could not be replaced by cystine, cysteine, thioglycollic acid or α-thioglycerol, and the rôle of this amino acid is obviously more specific than merely that of a source of organically combined sulphur.

The quantitative growth responses of *V. cyclosites* and *V. neocistes* to nicotinamide and methionine are shown in Fig. 1.

Preliminary study of biochemical properties

Adequate growth having been obtained in a medium containing mineral salts, nicotinamide and five amino acids, speculation as to the nature of energy-yielding reactions, and possibly of biosynthetic routes to other amino acids, led to investigation of the oxidation of the organic constituents of the medium and of the ability of the organisms to carry out the transaminase reaction. Using the Warburg technique, repeated experiments failed to demonstrate any oxygen uptake with thick washed suspensions; 6–8 mg/ml of both organisms were shaken with nicotinamide and each amino acid in turn, both in phosphate buffer and in the basal mineral medium. The addition of a crude preparation containing coenzymes I and II failed to promote oxygen uptake. Furthermore, it was not possible to demonstrate any evidence of transamination between the five essential amino acids and pyruvic acid or α-ketoglutaric acid. Although pyridoxine had been shown not to be essential for growth it was included in replicate transaminase experiments, but no evidence of transamination was obtained. The experimental procedure adequately demonstrated transamination using *Escherichia coli*, an organism known to carry out this reaction.

Discussion

A defined minimal medium which would support the growth in continuous subculture of both *V. cyclosites* and *V. neocistes* consisted of a mineral basal medium containing nicotinic acid or nicotinamide and the amino acids methionine, histidine, aspartic acid, phenylalanine and *iso*leucine. No additional source of carbon other than that supplied by the growth factors was necessary; presumably energy for growth must be obtained by the dissimilation of one or more of these compounds unless an autotrophic energy-yielding mechanism is postulated. Washed suspensions of both organisms failed to oxidize the organic components of the medium, nor was it possible to demonstrate transamination between the essential amino acids and α-ketoglutaric or pyruvic acid. It is not possible at this stage to decide whether washed suspensions of these organisms are inherently incapable of carrying out these two types of reaction or whether the washing process renders them so incapable. The process of washing bacteria is known to reduce their metabolic activity in certain cases because essential coenzymes diffuse from the cell. In the two reactions so far investigated, however, the addition of cofactors failed to promote activity. It is possible that these organisms are only metabolically active when growing, and that the washed non-growing suspension is without activity; such a hypothesis is not inconsistent with their general metabolic inertness.

Although the field of this investigation was basically nutritional and biochemical some doubt is left in the authors' minds as to the correct classification of the organisms used. Shewan *et al.* (1954) stated that all the vibrios tested by them were inhibited by Terramycin and compound O/129, but in the present work it was never possible to demonstrate inhibition by the latter although inhibition by Terramycin was always obtained. The failure to conform to the pattern set out for vibrios by Shewan *et al.* and the lack of comma-like forms may not, however, for the systematic bacteriologist, be obstacles to the assignment of these organisms to the genus *Vibrio*.

References

Breed, R. S., Murray, E. G. D. & Hitchens, A. P. (1948). *Bergey's Manual of Determinative Bacteriology*, 6th ed. London: Baillière, Tindall & Cox.

Gray, P. H. H. & Thornton, H. G. (1928). Soil bacteria that decompose certain aromatic compounds. *Zbl. Bakt.* (2 *Abt.*) **73**, 74.

Shewan, J. M., Hodgkiss, W. & Liston, J. (1954). A method of rapid differentiation of certain non-pathogenic asporogenous bacilli. *Nature, Lond.*, **173**, 208.

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Telling, R. C., Elsworth, R. & East, D. N. (1958). *J. appl. Bact.* **21** (1), 26–44.

A CONTINUOUS INFRARED ANALYSER FOR MEASUREMENT OF CO_2 IN EFFLUENT AIR FROM BACTERIAL CULTURES

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SUMMARY: The report reviews methods of continuous infrared absorption analysis and gives the description and performance of a commercial infrared CO_2 analyser. Examples are given of the use of an infrared analyser recorder to measure CO_2 in effluent air from deep cultures. This measure of metabolic activity can be used to estimate growth rate. It is particularly useful because results are obtained with little or no measurement lag and without the need to sample the culture fluid itself.

For the estimation of growth in liquid cultures, probably the most favoured indirect method is by measurement of the optical density of a series of samples. Compared with an estimate based on measurement of viability, this manual method is labour saving and yields a result quickly; but an automatic method of immediate and continuous measurement would be more effective and lay open the way, where desirable, to automatic process control. Optical density measurement is not always satisfactory for growth measurement, e.g. we have found the addition of antifoam to a culture sometimes causes transient changes in optical density which are not related to a corresponding change in bacterial density. Other indirect methods of examining a culture fluid, such as measuring growth by the consumption of a metabolite or by measuring the accumulation of a metabolic product, are chemical and not suitable for continuous measurement.

Where growth conditions such as aeration, pH value and agitation are controlled it is likely that continuous measurements by physical methods of the CO_2 produced or the O_2 consumed can be used to give an immediate estimate of growth rate, and have the advantages envisaged above. In addition the less frequent sampling of the culture itself, which will result from analysing the effluent air, will reduce the risk of contamination.

Apparatus for continuous analysis of both CO_2 and O_2 in gases can be bought. This report reviews the methods of continuous infrared absorption analysis, and gives a description and the performance of a commercial infrared CO_2 analyser. As well, examples are given of the use of the meter in microbiological work. The value of continuous CO_2 analysis for the general control of fermentations is also considered.

The paper is written by authors who have no special theoretical knowledge of infrared analysis and is a record of experiences with the first instrument commissioned. This model is now four years old and we are informed that the instrument

now supplied has many improvements which make for easier operation and greater reliability, features which are of importance when the machine is required for routine use.

Infrared Absorption Analysis

Critical reviews of continuous infrared analysers have been given by Devine (1951) and Hausdorff (1957*a*) (see also Mole, 1958). Other descriptions include those by Luft (1943), Fastie & Pfund (1947), Weissberger (1949), Martin (1953), Thomas (1954), Strouts, Gilfillan & Wilson (1955) and Jones (1956). All these sources have been used in the compilation of the descriptions which follow.

The better known method of infrared absorption analysis uses the spectrometer. The light beam after passing through the absorption cell is resolved into a spectrum which is scanned by a detector which measures the intensity of each wavelength or band of wavelengths. After comparing the intensity of the transmitted light with its incident intensity by using a 'blank' cell, the familiar graph is produced in which absorption is plotted against wavelength, the peaks in the curve indicating infrared absorbing substances in the sample. The usual range of wavelength examined in a spectrometer is from about 0·75μ to 25 μ.

When it is desired to analyse for only one component there is no need to use a spectrometer, and by arranging to pass a constant stream of the sample a continuous record can be made. Such instruments are usually referred to as non-dispersive continuous analysers and are in common use for gas analysis. The wavelength of the incident radiation is restricted to include a band at which the compound to be analysed absorbs. The transmission within this band is then compared with the incident intensity. This comparison may be made by either of two methods. One, due to Luft, is called the positive method, while the other is referred to as the negative method. Non-dispersive analysers are used to examine absorption in the range of wavelength 3 μ to 15 μ, nichrome wire at dull red heat forming a convenient radiation source.

Non-dispersive infrared analysers may be used for the analysis of many hydrocarbons, CO, CO_2, H_2O, N_2O, NO_2, etc. Because there is no monochromator to resolve the transmitted beam and because the amplifying system is thereby simplified, the infrared non-dispersive analyser is sufficiently rugged to be used on a plant and by non-specialists. Non-dispersive analysers are also cheaper than spectrometers. But an infrared analyser, since it measures absorption, will not necessarily give a correct answer if another substance present in the sample absorbs in the same waveband as the component it is wished to analyse, and in the case of a CO_2 analyser a variable quantity of water vapour in the sample will interfere in this way. This possible error is overcome by drying the sample before analysis.

The positive or Luft type non-dispersive analyser

This is shown in Fig. 1. The radiation is divided into two equal beams which, after passage through the holes in the rotating disc, reach the absorption cells C_A and C_R as pulses. The beams leave the cells and are directed on to selective detectors (T_A and T_R) which respond only to the same radiations as those which are absorbed

by the gas being analysed. The detector is made selective by filling it with the component to be analysed. Thus in a CO_2 analyser the detector contains a mixture of CO_2 and a gas such as argon which does not absorb in the infrared. As a result it will only respond to radiations in the wavebands in which CO_2 absorbs.

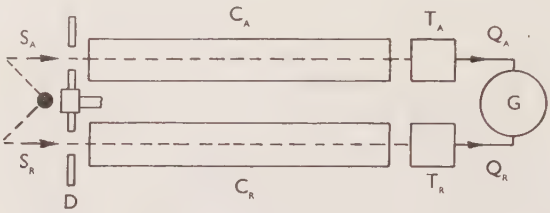

Fig. 1. The positive or Luft type analyser. S_A and S_R, analysis and reference beams; C_A and C_R, analysis and reference cells; T_A and T_R, analysis beam and reference beam detectors. The difference $(Q_R - Q_A)$ between the output signals Q_R and Q_A of the detectors is read on meter G.

The analysis beam S_A passes through the analysis cell C_A through which the dried gas being analysed flows. Here absorption takes place if there is CO_2 present, and also if there are substances which absorb in the same or different wavebands as CO_2. The beam S_A which is impressed on the selective detector T_A results in a signal Q_A which is a measure of the absorption by the CO_2 in the analysis cell and by any other substances which absorb in the same wavebands as CO_2. The signal is at a maximum when cell C_A contains no CO_2, and decreases when CO_2 is present.

The reference beam S_R passes through the reference cell C_R containing gas which in the case of a CO_2 analyser is free from CO_2 and is also dry. No infrared absorption will take place at those bands in which CO_2 absorbs and so the signal Q_R reaching the meter by this route will be constant and correspond to signal Q_A when the analysis cell contains no CO_2. The signal measured by the meter G is equal to $Q_R - Q_A$ and is proportional to the amount of CO_2 present in the analysis cell.

The negative type non-dispersive analyser

This is shown in Fig. 2. It differs from the positive type in three main features. First, there is no rotating disc in the light path, so that both incident beams are

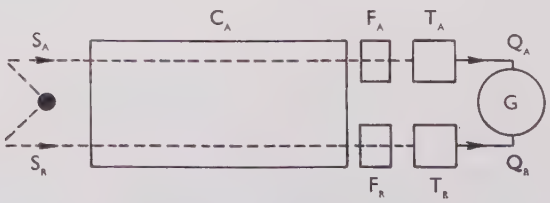

Fig. 2. The negative type analyser. S_A and S_R, analysis and reference beams; C_A, analysis cell; F_A, analysis filter cell (non-absorbing); F_R, reference filter cell; T_A and T_R, analysis beam and reference beam detectors. The difference $(Q_R - Q_A)$ between the output signals of the detectors is read on the meter G.

continuous. Second, there is no reference cell. Instead, its function is served by a gas filter cell. Third, the detectors are bolometers or thermopiles which are non-selective and respond to the whole spectral range of the incident radiation.

The analysis beam S_A passes through the analysis cell C_A in which the dried sample gas flows. Absorption occurs in C_A by virtue of the presence of CO_2 and also because of any other gas which absorbs infrared radiation in the same or other wavebands. The analysis beam S_A now passes through the gas filter cell F_A which is charged with a gas which does not absorb in the infrared (e.g. argon). The beam is then directed on to the non-selective detector T_A which is sensitive to all the radiation it receives. The subsequent signal Q_A transmitted to the meter measures both the absorption by any CO_2 present and by any other background gases which absorb infrared.

The reference beam S_R also passes through the analysis cell but in this case the filter cell F_R which follows is charged with CO_2 and absorbs the remaining radiation of the appropriate wavelength which has not been absorbed by the CO_2 present in the analysis cell. The signal Q_R from the non-selective detector T_R is therefore a measure of the absorption of only the background gas. The net signal $Q_A - Q_R$ read on the meter G is therefore proportional to the CO_2 content of the sample (plus any substances which absorb at the same wavelength).

Comparison of the types

For a critical review Hausdorff (1957) should be consulted. Both types will give incorrect results if another substance is present, in a variable concentration, which absorbs in the same waveband as the substance being analysed. To analyse another gas the Luft type is converted by changing the gas in the detector and the negative type by changing the gas in the filter, but it is doubtful whether any gas that may be analysed, other than CO_2, would be of interest in microbiology. The cancellation of the effect of other absorbing background gases is arrived at in different ways in each instrument. Electrically the Luft type is considered to be the more stable instrument of the two.

Interfering substances in the gas sample

Of the substances likely to be present in effluent air from a bacterial culture, oxygen, atmospheric nitrogen and hydrogen show no infrared absorption; but water, which will always be present, absorbs in the same wavebands as CO_2. The effect of the background gases (known as 'collision broadening') on the apparent infrared absorption of CO_2 may be neglected in this application because the background gas is air of substantially constant composition at all concentrations of CO_2 measured. Since absorption is a characteristic of the -OH bond, volatile alcohols in a culture medium may also interfere. Other substances, which may occur in bacterial cultures, with absorption bands in the same zone as CO_2 are acetone, acetaldehyde and substances containing -SH bonds. The most likely interfering substances are water vapour and, possibly, alcohol vapour. The effect of water vapour can fortunately be overcome either by standardizing with gas at the same humidity as the sample or by drying the gas before analysis and standardizing with dry gas.

The effect of water vapour and CO_2 in the reference gas of a Luft type CO_2 analyser

In a single scale instrument, traces of absorbing substances in the gas in the reference cell such as water vapour and CO_2 do not matter so long as the concentration is constant, because the absorption is allowed for in setting the instrument zero. In a multiscale instrument this is not necessarily so. In such instruments it is usual to place the analysis cells corresponding to each scale co-axially in the path of the analysis beam, and the two not in use at any one time are connected in series with the reference cell to form the flushing circuit. If the meter is standardized whenever the scale range is changed, then *constant* residual amounts of absorbing substances in the circulating gas are corrected for when setting the instrument zero. If, however, it is wished to change scale by merely re-arranging gas flows through the instrument without re-standardizing, then for greatest accuracy it is important that there are no residual absorbing substances in the flushing circuit gas.

As an example, in the instrument to be described (see Fig. 3) the effect of the small amount of water vapour present in the flushing gas if soda-lime is used as the purifier will be as follows. The A, B, and C scales measure from zero to 0·05%, 0·5% and 5·0% CO_2 respectively, and so the respective effective lengths of the analysis cells are in the ratio 100:10:1. When water vapour is present in the flushing gas, although the concentration (moles/unit volume) will be the same in each cell, the absorption due to water vapour which depends upon cell length will for scales A, B, and C be in the respective proportion of, say, $100x$:$10x$:x. Therefore if the analyser is adjusted at zero with CO_2-free gas in analysis cell C_A, then cells C_B and C_C which are in the flushing circuit will be absorbing from the analysis beam in proportion to $11x$ on account of water vapour in the flushing gas. If cell C_C is now made the analysis cell, cells C_A and C_B will be in the flushing circuit and will absorb from the analysis beam in the proportion of $110x$ due to water vapour in the flushing gas. As a result the reading which was previously at zero on scale A will now have a positive value on scale C. Any subsequent gas analysis made on scale C will be inaccurate to the extent of this change in zero. Table 1 shows qualitatively the expected error in analysis of a sample gas, due to water vapour in the flushing circuit, when after standardizing on one range a change is made to another.

Table 1. *The expected qualitative effect on the error of analysis due to water vapour in the flushing circuit of a multiscale Luft type analyser*

Zero set on scale	Changed to scale	Proportionate change in amount of water vapour in analysis beam	Expected change in zero	Expected error in analysis of a sample
A	B	$+90x$	Increase	Positive
	C	$+99x$	Increase	Positive
B	A	$-90x$	Decrease	Negative
	C	$+\ 9x$	Increase	Positive
C	A	$-99x$	Decrease	Negative
	B	$-\ 9x$	Decrease	Negative

A Commercial Infrared CO_2 Analyser

Description

The instrument to be described is the type SB/H supplied by the Infra Red Development Co. Ltd., Welwyn Garden City, Herts., and the subsequent limited description is based on the Company's handbook. The scale ranges are: A, 0–0·05%; B, 0–0·5%; C, 0–5·0%. The meter uses the Luft principle but the signal from the detector is not measured directly. Instead, it is used to operate a servo motor which adjusts a shutter which in turn controls the radiation leaving the reference cell until it matches the radiation from the sample cell. The shutter position is then a measure of the CO_2 content of the gas.

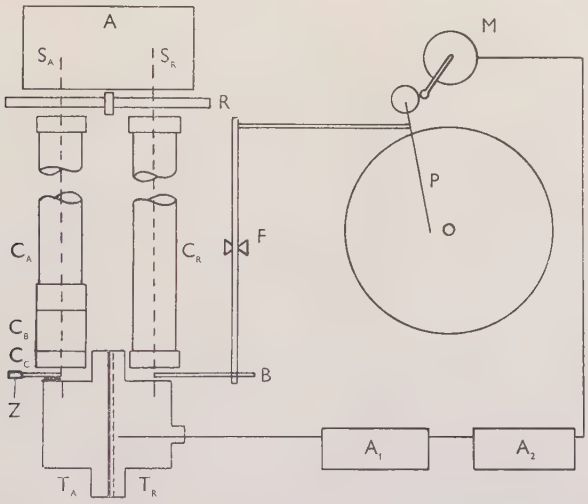

Fig. 3. The type SB/H commercial analyser. A, light source; S_A and S_R, analysis and reference beams; R, rotating shutter; C_A, C_B and C_C, analysis cells; C_R, reference cell; T_A and T_R, detector compartments; Z, zero shutter; B, balancing shutter; F, pivot point of balancing shutter operating arm; M, servo motor; A_1, A_2, amplifiers; P, pen arm.

Optical paths. Fig. 3 is a diagram of the instrument. The light source A is divided into two similar beams which are converted into pulses by the rotating shutter R. The analysis beam S_A passes, in turn, through the analysis cells C_A, C_B and C_C and past the zero shutter Z, which is used to equalize the effective intensity of the two incident beams. The analysis beam then enters compartment T_A of the selective detector, where the appropriate radiation energy is converted to heat. The reference beam S_R passes through the reference cell C_R, passes the balancing shutter B and enters compartment T_R of the detector, the appropriate radiations there being converted into heat.

The detector. The division between the two compartments T_A and T_R is made by a flexible diaphragm containing a capillary which allows the gas pressure on both sides of the diaphragm to equalize. The *detecting element* consists of a condenser, one plate of which is the flexible diaphragm and the other is a stationary metal grid placed near the diaphragm on the reference side of the detector. Another grid placed on the analysis side of the detector balances out the continuous electrostatic attraction between the two plates of the detecting element. The detector is charged to a fixed total pressure with a mixture of 5% CO_2 in argon to give a partial pressure of CO_2 of about 40 mm of Hg. We have found that a satisfactory total pressure is 79 cm of Hg. This ensures that even at the maximum barometric pressure encountered in this laboratory the detector pressure is always more than atmospheric—a practical aid when the detector needs re-charging. Because infrared absorption varies with temperature, the detector and the optical cells are each maintained by a thermostatically controlled heater at a constant temperature which is higher than the maximum ambient temperature.

The flushing circuit. The gas to be analysed is led into the required analysis cell through a 3-way tap. The same tap arranges that the two analysis cells not in use are connected in series with the reference cell. The flushing system is operated by a pump which draws air from the optical compartment and delivers through the purifier, through the reference cell and then through the two spare analysis cells and back into the optical compartment. In this way the optical paths inside the compartment are kept in similar condition to those in the reference and spare analysis cells.

Working principle. This can be understood by considering the effect of an increase in the CO_2 concentration of the gas being analysed. Immediately before the increase in concentration the radiation energy of the two beams entering the detector is equal, the balancing shutter having been adjusted by the servo motor until this is so. As a result, the pressure produced in each compartment during each short pulse is the same and the diaphragm is undeflected. No change is occurring in the capacity of the condenser, so the servo motor and the pen are stationary. Immediately the concentration increases, the radiations are unequal—that reaching the reference side is greatest. Since the thermal capacity of the detector gas is small, the pressure produced in the reference compartment during each pulse will be greater than that in the analysis side. In consequence the diaphragm will vibrate. This will be shown as a repeated change in the capacity of the condenser which is used as a signal to start the servo motor M. The direction of rotation is such that the movement of the balancing shutter reduces the amount of effective light in the reference beam until it is equal to the new value of the intensity of the analysis beam. As a result the signal from the condenser is extinguished and the pen arm P has taken up a new stable position corresponding to the new concentration of CO_2.

The scale reading is correct at normal atmospheric pressure (p_s=760 mm of Hg). Infrared absorption is proportional to the mass of absorbing molecules in the light path and is therefore proportional to the absolute pressure of the gas in the analysis cell. Thus if for an observed reading of Q_{obs}% CO_2 the barometer is p_o mm Hg, then the corrected reading (Q_{act}% CO_2) is equal to $Q_{obs} \times p_s/p_o$% CO_2.

The purification of gas in the flushing system

Standardization for one scale only. If it is decided to re-standardize whenever a change of scale is made, soda-lime (10–16 mesh) may be used by itself as absorbent. The bed is 5 in. long and 1 in. in diameter and is retained between cotton wool plugs to prevent solids being carried into the cells. The absorbent weighs about 40 g, and is changed every third day when about 60% exhausted.

Standardization for maximum accuracy on all scales. When scale changing without having to re-standardize is desired, the soda-lime tube should be followed by a 12 in. length of 1 in. diameter Pyrex pipe line containing 80 g of activated alumina (4–8 mesh, Peter Spence & Sons Ltd., Widnes) with a layer of indicating silica gel at the exit, and retaining plugs of cotton wool at each end.

Drying and metering the sample gas

The sample gas is passed at a controlled rate of 1 l./min through an air cooled condenser made from about 12 ft of $\frac{1}{4}$ in. bore copper tube of $\frac{3}{8}$ in. o.d. wound in a spiral, followed by a catchpot of about 1 l. capacity. The cooled gas is then dried by passing through a train consisting of: (a) two 12 in. lengths of 1 in. Pyrex pipe line each holding 80 g of activated alumina retained between cotton wool plugs, (b) one 6 in. length of 1 in. Pyrex pipe line holding 30 g of indicating silica gel also held between cotton wool plugs, (c) a micro filter to remove bacteria (Elsworth, Telling & Ford, 1955), (d) a rotameter (range, 0·2–2·0 l./min) followed by a tee-piece to which a water manometer (0–6 in. gauge) is connected, and (e) a sintered glass filter (10 μ particle size) connected to the analyser inlet by a well washed polythene tube in preference to natural rubber. The manometer is used to ensure that the supply pressure never exceeds 2 in. of water, and is normally isolated with a screw clip. The final connection of polythene tube is to minimize the chance of solids entering the analysis cells. The drying tubes need renewing every 6–12 hr.

Activated alumina and silica gel were chosen as drying agents highly suitable for routine use. In the dry state both desiccants adsorb CO_2 to a slight extent and give it up when wet. When high accuracy is required the degree of adsorption should be investigated and, if necessary, an absorbent such as magnesium perchlorate used instead.

Standardization

As will be shown later (under *Performance*) the proportional error is liable to be great if readings are taken towards the zero end of a scale. Thus, if it is desired to make measurements of an increasing magnitude (e.g. effluent air from a batch culture) then to get the greatest accuracy it is desirable to be able to make continuous measurements on successive scales during the course of a single experiment. If a drier is inserted in the flushing circuit this can be done, because then re-standardization is not necessary at a scale change. In other instances observations on only one scale may be needed. In such cases soda-lime, alone, is a satisfactory flushing circuit purifier.

Standardization for maximum accuracy

Having ensured that the absorbent in the flushing section is in good order and that the detector is correctly charged, the meter should be switched on for at least one hour, and preferably overnight, to become stable. It is best to adjust for zero on the most sensitive range (scale A) by passing CO_2-free nitrogen, dried as described, into the corresponding analysis cell. If the instrument is otherwise in order, the pen arm is then adjusted to read zero by moving the zero shutter which controls the intensity of radiation in the analysis beam.

Standard gases are analysed by other methods (e.g. chemical absorption). Thus the analytical error on a standard gas suitable for use on scale C will be less, in terms of percentage of full scale instrument reading, than on the other two scales. Hence scale C should be chosen for adjustment of scale reading, to obtain the best accuracy of standardization.

A gas containing $Q_{act}\%$ CO_2 corresponding to 60–70% of full scale reading is passed through the analysis cell (C_C). When a steady reading is obtained, if the observed reading Q_{obs} differs from $Q_{act} \times p_o/p_s$ then the pivot point F (Fig. 3) of the arm operating the balancing shutter B is adjusted until the reading is correct. As this involves opening the case, the internal temperature will change and so time must be allowed to regain stability before taking further readings.

Standardization for use of one scale only is carried out in a similar manner by adjusting zero and scale reading on the range it is proposed to use.

Routine calibration

We have made a practice of calibrating the meter about twice weekly with the standardizing gas. The error is plotted on a quality control chart (Tippett, 1952) on which the control limits are set at $\pm 1\%$ of full scale. This value is based on that of the reproducibility, which is later shown to be, for practical purposes, $\pm 1{\cdot}0\%$ of full scale at the 95% confidence interval. At these limits it can be expected that by chance about five in every hundred samples will fall outside the limits. When correcting action is deemed necessary, before adjusting the instrument other more likely causes such as a defective drying train on the sample line, or a breakdown of the flushing circuit purifier (which will also produce a zero drift), should first be examined. Failing a solution from these causes, instrument faults may then be considered, after first ensuring that the thermostat controlling the detector heater is working properly.

Performance

In these results the term *reproducibility* (in preference to the chemist's term *precision*) is used for the measure of agreement between replicate analyses. It is expressed as the limits at a given probability within which replicate observations will lie. In the same way *accuracy* of an observation is given by the limits at a given probability within which the true value lies. *Stability* is a measure of the period of time in which replicates of a measurement do not show any significant change. The estimates of these quantities are measures of the cumulative errors arising from factors such as: (a) condition of the reflecting surfaces in the infrared source, (b) condition of the

windows in the optical paths, (c) maintenance of gas in the detector, (d) temperature of the detector, and (e) performance of the servomechanism.

Reproducibility. The flushing gas was fully dried by the inclusion of activated alumina in the circuit. Immediately after standardizing with a gas containing 2·92% CO_2, cylinder samples ranging from 0·03% (air) to 4·0% CO_2 were successively measured on the appropriate scale at intervals of a few minutes, without making any standardizing adjustments. After each sample had been passed, the analysis cell was swept out with dry CO_2-free air until the reading was constant. The results, corrected for barometric pressure, are given in Table 2.

Table 2. *The results of reproducibility tests with a Luft type analyser*

CO_2 in standard gas (%)	Scale	Analyser reading (% full scale) in expt							Variance
		1	2	3	4	5	6	Mean	
0·03	A	57·2	57·4	58·2	58·8	58·2	57·6	57·90	0·36
0·65	B	12·4	12·6	12·6	12·6	13·0	12·6	12·63	0·036
0·40	B	78·6	78·6	79·2	79·6	78·6	79·6	79·03	0·25
0·40	C	8·34	8·34	9·36	8·96	9·16	9·16	8·89	0·193
2·92	C	58·6	58·4	58·2	58·2	58·2	58·8	58·40	0·063
3·90	C	78·6	79·0	78·6	78·6	79·0	79·0	78·80	0·048
								Mean	0·16

A correlation cannot be shown between the variances of observations at different points on the scales and their means, nor is it evident from the distribution of the variances that they are from other than a single population. It is therefore reasonable to describe reproducibility by the mean value of variance (0·16) which corresponds to a standard deviation of 0·4% of full scale. Therefore the reproducibility, which is the range in which replicate analyses can be expected to fall, is about ±1% of full scale at the 95% confidence interval. Thus, up to 0·05% CO_2 the reproducibility at this interval can be expressed as ±0·0005% CO_2 (±5 parts/10^6); from 0·05–0·5% CO_2 as ±0·005% CO_2 and from 0·5–5·0% CO_2 as ±0·05% CO_2. In other words, on the A and B scales the reproducibility approaches that to be obtained from gravimetric chemical analyses of quite large samples. On the C scale, reproducibility is better than that of the Orsat apparatus and approaches that of the Haldane apparatus.

It can be seen that observations at the zero end of a scale should be avoided because they are subject to a high proportional error. Thus, if it is required to make measurements of satisfactory accuracy over a range of more than 5:1, a multiscale instrument is desirable.

Accuracy. As defined above the accuracy A_m depends upon two factors, the reproducibility of the instrument R, and the accuracy A_s of the analysis of the gas used for standardizing. It can be shown that $A_m = \pm\sqrt{\{2(R^2)+A_s^2\}}$ where all the values are expressed at the same confidence interval. Having measured reproducibility, accuracy can be calculated; and the function of a check on accuracy is to observe if actual performance is equal to that expected. The value of R has been approximated to ±1% full scale at the 95% confidence interval. The accuracy of

the standardizing gas supplied by the Infra Red Development Co. was given as 2·92% CO_2 ±3% (and assumed to be at the 95% confidence interval), which is equal to 2·92±0·088% CO_2 or, in terms of full scale reading, 59·4±1·8% when measured on the C scale. Therefore $A_s=\pm 1{\cdot}8\%$ full scale, so that $A_m=\pm\sqrt{\{2\,(1^2)+1{\cdot}8^2\}}=2{\cdot}3\%$ full scale at the 95% confidence interval.

In testing to find whether these limits are achieved in practice, it is clear that the accuracy of the analysis of the test gas must be considered. Thus when a meter of accuracy A_m is used with a test gas whose accuracy of analysis is A_t, the limits L_m within which the meter reading will fall are given by $L_m=\pm\sqrt{\{2\,(R^2)+A_s^2+A_t^2\}}$.

Table 3. *The results of accuracy tests with a Luft type analyser*

Scale	A	B	C
Analysis of standard gas (% CO_2)	0·03	0·39	4·0
Test gas accuracy (A_t) (% full scale)	0·0	±2·3	±2·4
Calculated limits (L_m) of meter reading (% full scale)	±2·3	±3·2	±3·3
Expected meter reading (% full scale)	60±2·3	78±3·2	80±3·3
Time after standardization (days)	Meter reading minus expected nominal value (% full scale)		
1	+1·0	+1·4	−0·6
2	+1·2	+1·4	−0·4
3	+2·0	+2·0	−0·6
4	+2·0	+2·4	−0·6
5	+2·0	+1·4	−0·2
6	+1·4	+2·4	−0·2

Checking was carried out using gases to give readings at the upper end of each scale. The A_t values of each gas were known, and from them L_m values were calculated. In the tests the gas in the flushing circuit was fully dried by having an alumina drier after the CO_2 absorbent. The meter was standardized on scale C, and without further adjustment the three test gases were measured on six successive days. The results obtained, corrected for barometric pressure, are given in Table 3. They show that, without exception, the meter readings for each gas fell inside the expected limits, showing that the accuracy achieved reached the expected value even when a test was made on a scale other than that upon which the meter was calibrated.

Stability. Repetitions of the tests reported in Table 2 over the two years the meter has been in regular use have shown no deterioration in reproducibility. Table 3 shows that accuracy was maintained over a period of 6 days. Over a test period of 45 days, however, a significant change was observed. In this test the meter, after initial standardization, was used continuously without further adjustment. A standard gas containing 4·0% CO_2 was analysed every second or third day; the results are given in Fig. 4. This shows that the observed value increased significantly

with time at an average daily rate of 0·1% of full scale reading. At the 45th day the detector was recharged with gas to the original pressure but no standardizing adjustments were made. The subsequent readings show how accuracy was regained. Gas leakage from the detector is typical of this particular instrument, and accuracy could generally be re-established by re-filling the detector. We are informed that significant detector leakage was not typical of the model at this stage of its development and is in fact repairable. Detector leakage in all current models is stated to be insignificant.

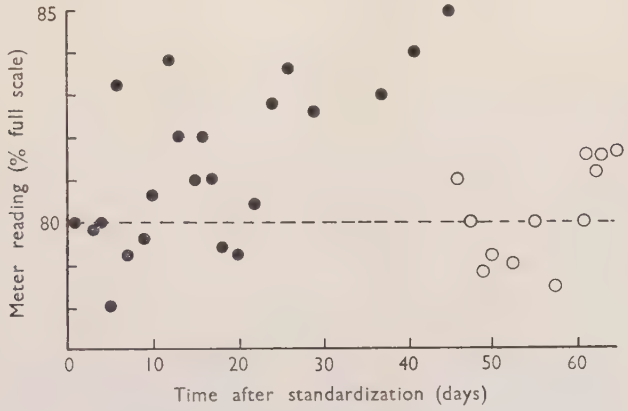

Fig. 4. The results of a stability test, showing how in time the meter reading drifted away from the true value. The estimate of the coefficient of correlation between time and error is +0·6, which is highly significant ($P<0·01$). Until the 45th day the average increase in error was 0·1% of full scale reading/day. At this point the error was corrected by refilling the detector.

Reports on the stability of other instruments are of interest. In the discussion which followed the paper given by Hausdorff (1957*b*), the latter stated that the period for which zero drift is less than 1% (presumably of full scale) should be at least a week but may extend to several months. Dr. Voetelink of Phillips Petroleum Co., speaking of several years' experience of infrared analysers, said that the main problems are not electronic or mechanical stability but correct preparation of the sample gas. He added that regular cleaning of the analysis cell is necessary. The present authors, when analysing culture vessel effluent air and using the purification train specified, have not found it necessary to clean the analysis cells. We do, however, place great importance on using clean, particle-free polythene tube for the final connection.

Reliability. Wall (1957) has said that when a process analyser does not receive the dedicated care of one responsible individual it must have a stability, reliability and ruggedness far exceeding that of a laboratory instrument. This aptly specifies an instrument suitable as an adjunct in research when used by workers who, like the writers, have had no formal training in electronics. The failure rate of the three

infrared analysers we have in use, after allowing for an installation period, averages about 6 months/instrument. It is interesting to note that this is similar to a value quoted by Taylor (1954) for the various electronic instruments used by the United Kingdom Atomic Energy Commission, which are cared for by specialist instrument groups.

The effect of water and ethanol vapour

Water vapour in the flushing circuit. A qualitative prediction of the effect of water vapour in the flushing circuit is given in Table 1. Table 4 gives the results obtained when using soda-lime by itself in the flushing circuit purifier, when there would be a residue of water vapour in the circulating air. The analyser was standardized by setting the zero with CO_2-free gas and adjusting the scale reading using gas containing 2·92% CO_2. A series of dry standard gases in cylinders was then analysed on scale C and also on scales A and B. A change was made to scale A and the zero adjusted to read correctly when analysing CO_2-free gas. Without further adjustments the same gases were then tested, on scales A, B, and C. Finally, a change was made to scale B, zero reading was adjusted to be correct and again without further adjustments the series of gases was tested on all scales. It will be seen that the experimental values (taking into account reproducibility) agree with the predicted values; and confirm that, if the flushing circuit gas is not dry, scale changing without re-standardization will result in significant errors.

Table 4. *Measurement of the effect of water vapour in the flushing gas on the meter reading of a Luft type analyser*

Scale, and expected reading (% full scale)	A, 60·0		B, 13·0		B, 80·0		C, 8·0		C, 58·4		C, 78·0	
	Errors		Errors		Errors		Errors		Errors		Errors	
Standardized on scale	E*	M†	E*	M†	E*	M†	E*	M†	E*	M†	E*	M†
C	−	−4·0	−	+0·4	−	+0·4	nil	+0·8	nil	nil	nil	+0·8
A	nil	nil	+	+1·0	+	+1·0	+	+1·8	+	+2·6	+	+3·2
B	−	−2·0	nil	nil	nil	−0·2	+	+1·2	+	+1·6	+	+0·2

* E, expected, see Table 1. † M, measured, in % full scale.

Table 5. *Errors in the meter reading of a Luft type analyser due to water vapour in the sample gas*

Scale	% full scale reading		Error due to undried gas (% full scale)
	Dry gas	Undried gas	
A	60	68	+8
B	6	8	+2
C	0·6	1·6	+1

Water vapour in the sample gas. Dry air (0·03% CO_2) was analysed on each scale. The same air was passed through a culture vessel containing distilled water at room temperature followed by the copper coil condenser and catchpot, and was then

analysed on each scale. The results (Table 5) show that the error as a percentage of full scale was pronounced on scale A, just significant on scale B, and negligible on scale C.

Ethanol vapour in the sample gas. A comparison was made between dry CO_2-free nitrogen, CO_2-free nitrogen saturated with water vapour at room temperature, and CO_2-free nitrogen saturated with aqueous ethanol (sp. gr. 0·990 at 15°/15°, chosen to simulate an alcohol from yeast fermentation). The readings on scale A were 0%, 6% and 8% full scale respectively, i.e. the effect of ethanol vapour was less than that of water vapour alone and, while just significant on scale A, was negligible on the other two scales.

Summary of performance

The reproducibility of the analyser was about ±1% of full scale at the 95% confidence interval. After calibrating on scale C with a standard gas supplied by the maker, the accuracy on all scales was about ±2% full scale at the same confidence interval. Stability was satisfactory in a 6 days test. Over a 45 days test there was a significant gradual deterioration, inferred to be due to leakage of gas from the detector. The reliability of a group of three analysers has been about 6 months/instrument/failure.

Water vapour in the sample gas can result in significant errors, but the effect of ethanol vapour can for most purposes be neglected.

In extension of the statement on accuracy, the scale-changing facility must be used with caution. If only soda-lime is used in the flushing circuit, then to secure maximum accuracy each scale must be individually standardized. However, by using a perfectly dry gas in the flushing circuit, scale changing may be made without re-standardizing and with maximum accuracy.

The Use of CO_2 Analysis

Measurement of generation time in a batch culture

Fig. 5 gives the results obtained with a deep culture of *Klebsiella cloacae* (*Aerobacter cloacae* NCTC 8197) in a phosphate-buffered glycerol medium. It shows the correspondence between estimates of growth rate in the logarithmic phase by measurement of the optical density of samples of culture taken periodically with those derived from continuous analysis of the CO_2 in the effluent air.

Optical density was measured on a single cell absorptiometer. The culture samples were diluted to give readings between 0·3 and 0·4 on the optical density scale, where the reproducibility was at its best and amounted to about ±3% of the observed reading, at the 95% confidence interval. The scatter of the optical density values around the regression line in Fig. 5 is compatible with this level of discrimination. The type SB/H infrared analyser was used to measure the CO_2. In this example the volume of effluent air was practically equal to that of the inlet air so that the % CO_2 is proportional to the amount of CO_2 produced.

The slopes of the regression lines of $\log_{10}$ (% CO_2) and $\log_{10}$ (optical density) against time do not differ significantly. The doubling time by optical density

measurement was 69 min and by CO_2 measurement 66 min. The estimate by CO_2 analysis is more precise, being (at the 95% confidence interval) within ± 2 min compared with ± 8 min by the use of the single cell absorptiometer. This result can be expected, taking into account that the CO_2 analyser has a better reproducibility and in effect examines an infinite number of samples.

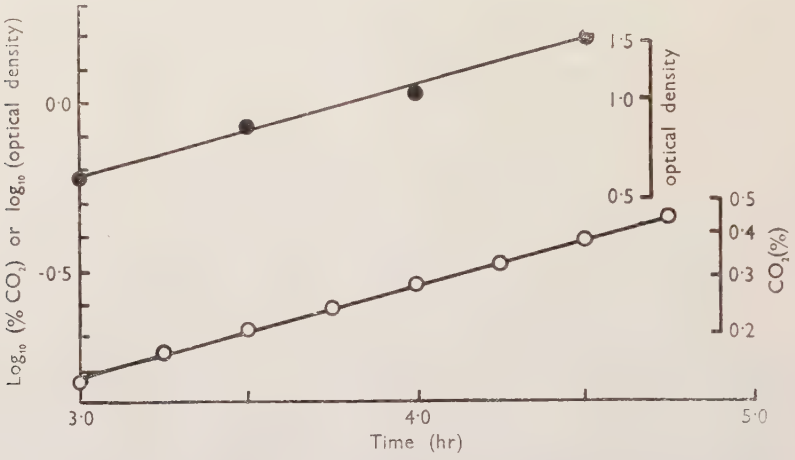

Fig. 5. The correspondence between estimates of growth rate in a batch culture of *K. cloacae* by measurement of optical density (full circles) and by analysis of the CO_2 in the effluent air (open circles).

The measurement of growth rate in a continuous culture with controlled pH

The distribution of CO_2 between the effluent air and the culture medium in a continuous culture differs from that in a batch culture. In the former, in the steady state, the concentration of CO_2 in the medium will be in equilibrium with the CO_2 in the effluent air. Depending on the pH value of the culture and the CO_2 in the effluent air, there will be a measurable amount of combined CO_2 in the outflowing culture fluid. This can be predicted theoretically from the Henderson-Hasselbach equation, which relates carbon dioxide pressure in the gas phase, bicarbonate ion concentration in the culture and pH value of the culture. In a batch culture, during the phase of maximum growth bicarbonate ions will accumulate in an endeavour to reach equilibrium with the CO_2 present in the effluent air. However, as growth reaches completion and CO_2 formation stops, the consequent reduction in the amount of CO_2 in the effluent air will result in a reduction in the bicarbonate content of the culture to nearly zero, in order to reach equilibrium with normal air containing 0·03% CO_2. The culture will now contain a residue of carbonate ion, the concentration of which will depend on the peak value reached by bicarbonate ion in the stage of maximum growth. If the pH value of the culture falls to 5·0, no bicarbonate or carbonate ion can exist, and so with continued aeration the amount of CO_2 measured in the exit air (above the normal value of 0·03% CO_2) will effectively

represent all the CO_2 formed during growth. In both types of culture at pH values greater than 5·0, an increasing amount of combined carbonate will exist, but the ratio (CO_2 in effluent air) : (residual CO_2 in the culture) will always be greater in a completed batch culture than in a continuous culture. Thus CO_2 in effluent air from a batch culture should theoretically be a better estimate of growth rate (or total growth) than the same measurement in a continuous culture.

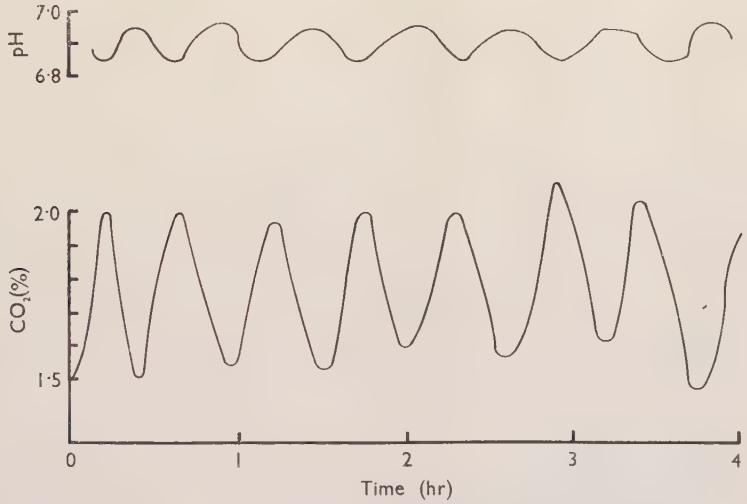

Fig. 6. The relation between the pH value of a continuous culture of *K. cloacae* and the CO_2 in the effluent air, when control of pH is by two-step action.

Table 6. *Details of a 20 l. continuous culture of* K. cloacae *with two-step pH control*

Flow rate of culture (l./hr)	Growth rate g dry cells/(l.)(hr)	CO_2 in effluent air (%)		Rate of CO_2 in effluent air mM/(l.)(hr)	Range of pH value*
		Range	Mean		
5	1·05	1·4–1·8	1·7	31·0	6·45–7·10
6	1·19	2·0–2·5	2·2	35·4	6·95–7·05
8	1·62	2·42–3·42	2·86	45·6	6·95–7·05
9	1·92	2·86–3·91	3·28	52·4	6·90–7·10
10	1·80	2·94–3·55	3·23	51·6	6·90–7·10

* Desired pH value, 7·0.

The situation in a continuous culture is more complex if one considers the result of applying two-step pH control. In this, when the measured value deviates from the desired value acid or alkali is admitted to the culture at a fixed rate. When the desired value is re-established the supply of acid or alkali is cut off. It is characteristic of this type of control that the measured value oscillates about a mean, the amplitude of oscillation depending on factors such as: (i) the transmission characteristics of the detecting element, (ii) the rate of change of the measured value, which in this instance

is a function of growth rate, (iii) the buffer system employed and the desired pH value chosen, and (iv) the rate of addition of alkali or acid. This oscillation in pH value, as the Henderson-Hasselbach equation predicts, can be expected to result in an oscillation in the bicarbonate content of the culture, finally reflected in an oscillation in the CO_2 content of the effluent air. This does in fact happen as shown in Fig. 6 and shows a limitation of two-step pH control.

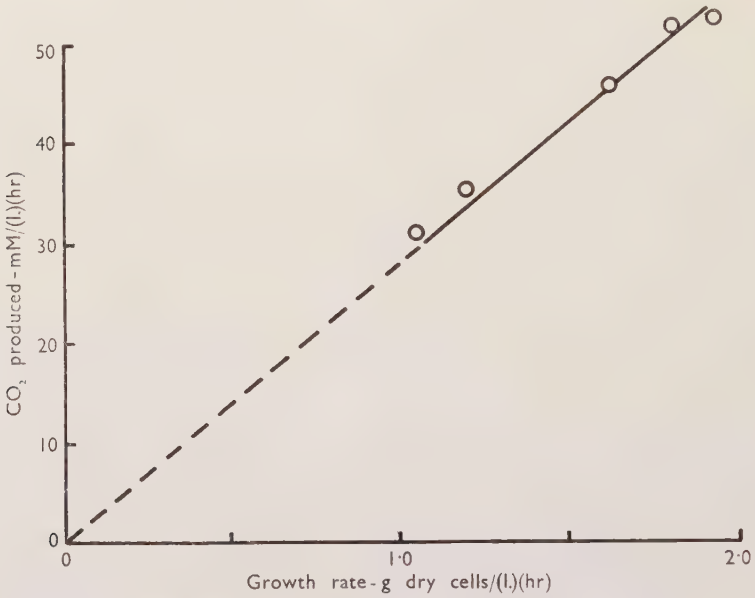

Fig. 7. The relationship between growth rate and CO_2 production by *K. cloacae* in continuous culture (see Table 6). The estimate of the correlation coefficient is 0·996, which is highly significant ($P<0·01$).

Nevertheless it is still possible to use CO_2 measurement as an estimate of growth rate, as Table 6 and Fig. 7 show. *K. cloacae* (NCTC 8197) was grown at 37° in a defined medium containing 10 g/l. glycerol, 0·05 M phosphate (as ammonium salt) and ethylenediaminetetra-acetic acid as a sequestering agent for the trace elements. Oxygen in the effluent air was measured on a Pauling paramagnetic analyser. Knowing the rate of inlet air to the culture vessel, the mean value of % CO_2 was used to calculate the rate of CO_2 in the effluent air. Fig. 7 shows a satisfactory correlation between these values and growth rate [(cell concentration × flow rate) ÷ volume of culture in the vessel. Units; g dry cell wt/(l.)(hr)].

Sorbose formation

Fig. 8 shows the possibilities of using CO_2 measurement to determine the progress of the conversion of sorbitol to sorbose by cultures of *Acetobacter suboxydans*. It shows how the completion of the conversion can be determined without resort to sorbose analysis.

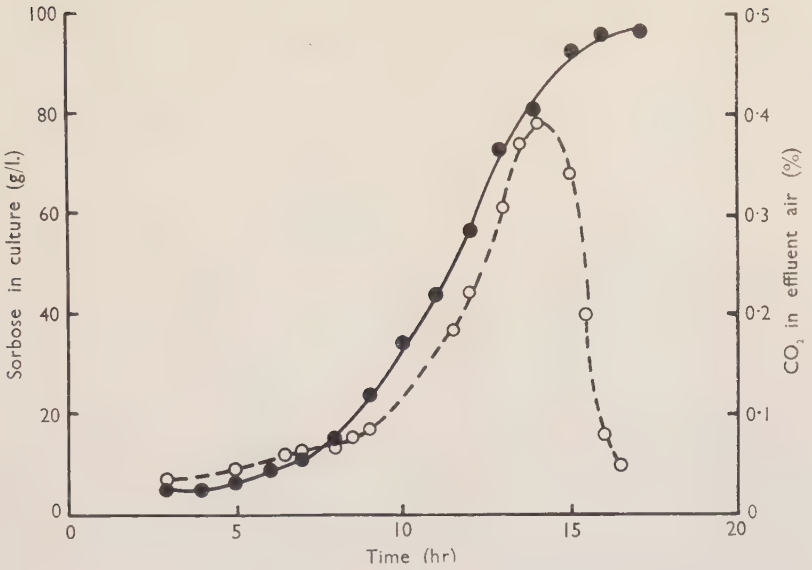

Fig. 8. The relationship between sorbose production from sorbitol (full circles) and CO_2 in the effluent air (open circles) for a batch culture of *Acetobacter suboxydans*.

Discussion

Most processes are operated by controlling certain variables at values which, experience has shown, produce the required quality of product at the maximum rate. The concentration and purity of products from purely chemical and physical processes can frequently be measured by physical methods. It is thus possible to maintain a frequent or even continuous check on product quality with a minimum of measurement lag. Information about a biological process cannot generally be obtained so quickly. At the best, for instance in the case of an extracellular product determined chemically or physically, there is the measurement lag of a laboratory analysis. At the worst, if bio-assay is the method employed, then 16–24 hr may elapse after sampling before a result is known. As well, and this applies more particularly to experimental work, such analysis is usually carried out by laboratory staff working only during the day: sampling therefore tends to be concentrated within that period. Thus there is frequently a gap of 15–18 hr each day during which there is no information. Control, as such, is therefore nominal, information calling for changes in cultural conditions often being received when it is too late to apply. In addition, transient changes which occur in the periods when sampling is infrequent may go unnoticed.

The additional use of infrared CO_2 analysis, accompanied by recording, removes or reduces these disadvantages. The examples show that effluent air analysis gives an *immediate* estimate of growth rate in both batch and continuous cultures. As is well realized, the CO_2 in effluent air is not always a measure of all the CO_2 produced by an organism. Under certain conditions effluent air analysis is therefore

not as suitable for estimation of total mass of growth as it is for growth rate. Of the organisms we have studied, for a given condition, each exhibits a characteristic CO_2 picture, which there is no reason to suppose does not apply generally. When growth rate can be determined without measurement lag, as CO_2 analysis permits, the effect of varying other process variables can be studied with much greater facility than when relying on bio-assay alone. The commanding view of growth rate which is given by continuous CO_2 analysis also enables better use of laboratory time by restricting the examination of samples to those likely to be most informative. Another aspect of continuous CO_2 analysis is that since it shows up a growth change immediately, when one occurs, the information invites immediate examination of other parts of the process for a likely cause. As a result we have found that a CO_2 analyser tends to improve supervision by routine workers.

We are indebted to Mr. S. Peto for his statistical analysis of the results. We wish to thank Mr. W. B. Bartley of the Infra Red Development Co. Ltd., and our colleague Dr. K. P. Norris for reading the manuscript dealing with the operation of the analyser; and Dr. D. Herbert for helpful criticism of the section dealing with pH-controlled continuous culture.

References

Devine, J. M. (1951). Application of infrared gas analysers. *Instruments* **24**, 1296.

Elsworth, R., Telling, R. C. & Ford, J. W. S. (1955). Sterilization of air by heat. *J. Hyg., Camb.*, **53**, 445.

Fastie, W. G. & Pfund, A. H. (1947). Selective infrared gas analysers. *J. opt. Soc. Amer.* **37**, 762.

Hausdorff, H. H. (1957*a*). Application of infrared continuous analysers. *Chem. & Ind.* (*Rev.*) no. 6, p. 154.

Hausdorff, H. H. (1957*b*). Application of infrared continuous analysers. *Chem. & Ind.* (*Rev.*) no. 7, p. 200.

Jones, E. B. (1956). *Instrument Technology*. Vol. II, p. 93. London: Butterworths Scientific Publications.

Luft, K. F. (1943). A new method of recording gas analysis by absorption of infrared radiation without spectral dispersion. *Royal Aircraft Establishment Translation No.* 109 (from *Z. tech. Phys.* **24**, 97).

Martin, A. E. (1953). The infrared gas analyser and what it can do. *Research, Lond.*, **6**, 172.

Mole, J. P. (1958). Operating experiences with non-dispersive infrared analysers. In *Automatic Measurement of Quality in Plant Processes*. London: Butterworth's Scientific Publications.

Strouts, C. R. N., Gilfillan, J. H. & Wilson, H. N. (1955). *Analytical Chemistry. The Working Tools*. Vol. II, p. 849. Oxford: Clarendon Press.

Taylor, D. (1954). The reliability of nucleonic instruments. *J. Brit. Instn Radio Engrs* **14**, 570.

Thomas, H. A. (1954). The automatic indication and recording of minute concentrations of organic gases in air. *J. Brit. Instn Radio Engrs* **14**, 527.

Tippett, L. H. C. (1952). *Technological Applications of Statistics*. p. 21. London: Williams & Norgate, Ltd.

Wall, R. (1957). Process infrared analysers – 1957. *Industr. Engng Chem.* (*Industr.*) **49**, 69A.

Weissberger, A. (ed.) (1949). *Technique of Organic Chemistry*, 2nd ed. Vol. I (Part 2), p. 1426. London: Interscience Publishers, Ltd.

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Fliegelmann, J. (1958). *J. appl. Bact.* **21** (1), 45–46.

A NEW PLUG FOR CULTURE FLASKS

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The practice of closing culture flasks with plugs made of cotton wool has certain disadvantages, especially when applied to large containers such as Roux bottles. The tightness and fit of the plugs present a problem because of the bulk of cotton wool required and the handling of such a plug is somewhat inconvenient. These factors are especially noticeable when many flasks are handled. The use of screw-caps, while partly eliminating these drawbacks, nevertheless presents other problems. Standard Roux bottles are not equipped with a thread for screw-caps, free exchange of gases in the flasks is hindered, while when flasks are handled, touching the neck with the palm of the hand is unavoidable. In order to solve these difficulties a convenient stopper has been devised, which may be used and sterilized repeatedly. Several hundreds of these stoppers have been used successfully in this Institute for over a year.

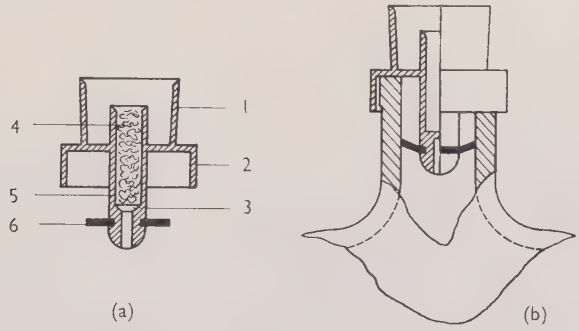

Fig. 1. Details of the new plug for culture flasks. (a) Vertical section. (b) Part section and elevation of the plug in position on the flask.

Construction

Details of the construction of the stopper are shown in Fig. 1. It is made of heat resistant material which must not react with the contents of the flask. Materials used are aluminium with anodic protection or 'Teflon', a plastic which withstands temperatures up to 500°F and is unaffected by acids and most alkalis. The shape of the stopper's head (1, Fig. 1a) permits an easy grip between the small finger and the palm of the hand, leaving the remaining fingers free. The broadest part of the stopper (2), which fits over and around the rim of the bottle, protects the rim

from contamination by the hand, or contamination of the hand by spilled contents of the bottle. The inside diameter of the central tube (5) is smaller in its lower section (3). This produces a seat, which prevents the cotton wool plug (4) from falling into the flask and at the same time permits ventilation and exchange of gases. A neoprene or silicone rubber washer (6) tightens the stopper to the neck of the bottle (see Fig. 1b).

Features

This stopper may be removed or replaced in one movement. The cotton wool plug (4) is easily exchanged (as in a pipette), and it ensures that no pressure or vacuum will build up in the bottle during autoclave sterilization. The protective ring (2) of the stopper allows clean and safe work. The neoprene washer may be renewed, if necessary, when washing the flasks. The use of a heat resistant silicone rubber washer avoids the need for frequent renewals.

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Franklin, J. G., Williams, D. J., Chapman, H. R. & Clegg, L. F. L. (1958). *J. appl. Bact.* **21** (1), 47–50.

METHODS OF ASSESSING THE SPORICIDAL EFFICIENCY OF AN ULTRA-HIGH-TEMPERATURE MILK STERILIZING PLANT. II. EXPERIMENTS WITH SUSPENSIONS OF SPORES IN MILK

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SUMMARY: The sporicidal efficiency of an ultra-high-temperature (UHT) milk processing plant has been tested using spores of a strain of *Bacillus subtilis* in milk. With the inoculum and volume of milk adjusted to obtain a countable number of survivors by a conventional dilution counting method, a temperature of 130·5° with the minimum time setting of the plant was found necessary to give a destruction of 99·99999%. This temperature was lower than that found previously (135°) for spores suspended in water and evidence is produced to support the suggestion that UHT milk may be inhibitory to the germination and/or subsequent growth of heated spores.

The advantages of sterile milk produced by an ultra-high-temperature (UHT) process will not be fully utilized until this milk can be filled aseptically into sterile bottles. A temporary alternative already being practised is to follow the UHT treatment with an in-bottle treatment to destroy contamination from the filler, capper and container, but this treatment results in certain changes in the colour and flavour of the UHT milk even if the in-bottle treatment is less severe than normal (Williams *et al.* 1955).

Williams *et al.* (1957) suggested that a spoilage rate of not more than 1 pint bottle in 1,000 would be necessary to satisfy trade requirements. To achieve this figure it was considered that a destruction of spores in the milk alone of the order of 99·99999% must be achieved. This would allow a reduction in the spore content from the average figure of 1/ml in raw milk observed by Franklin *et al.* (1956) to not more than 1 spore/1,250 gal in the treated milk, and would still allow some latitude in the bottling process.

The present work was carried out to determine the temperature at which this degree of destruction would be most conveniently achieved.

Experimental Methods

The plant tested was an A.P.V. HX-type heat exchanger installed in the N.I.R.D. dairy; the layout and operation have been described by Williams *et al.* (1957). The two filter assemblies were replaced by a diversion point and also a modification of the sampling cock of the type described by Williams *et al.* (1955). This cock was fitted with a threaded outlet instead of Jubilee clips to carry the outlet shroud.

The plant was sterilized with steam at 40 lb/in.^{2} for 30 min using the air filter to prevent re-contamination (Williams *et al.* 1955). The plant was run on inoculated milk instead of water as in previous work.

Test organism

Spores of a strain of *Bacillus subtilis* (786) isolated by Franklin *et al.* (1956) were used, the spore suspensions being prepared in a similar way to that described by Williams *et al.* (1957). However, a modification in the sampling and testing methods increased the number of spores required beyond the capacity of an ordinary bacteriological laboratory. A supply of 3×10^{14} spores was obtained, therefore, from the Ministry of Supply, Microbiological Research Establishment, Porton, Wilts.

Bacillus subtilis (786) exhibits marked heat activation and it was necessary to allow for this when calculating the experimental inoculation levels.

Sampling, and operation of the plant

The method of sampling by membrane filter used with spores in water (Williams *et al.* 1957) was unsuitable, because the membranes could not filter milk. The churn method previously used was also unsuitable because greater precision in the determination of end-points was required than that afforded by a positive or negative result. It was considered that the best method of determining the actual number of survivors in the milk after treatment was by a dilution count method. To obtain end-points by dilution counts, with a destruction of spores of 99·99999%, required the use of a large inoculum in the raw milk to avoid the sampling of very large quantities of treated milk. Accordingly, 20 gal of raw whole milk were inoculated with spores of *B. subtilis* 786 to give a final concentration of *c.* 5×10^6/ml and a sample of *c.* 2 litres of treated milk was collected in a sterile 10 l. jar. (A method for assessing the bacteriological effect of sterilizing processes on raw milk inoculated with large numbers of spores was described by Galesloot in 1956).

Previous work with spores in water had indicated that a temperature of *c.* 135°, with the variable holding tube set at its minimum position, gave a spore destruction of 99·99999%. Treatment temperatures of 130·5°, 132·0°, 133·5° and 135·0° were therefore tested with the tube set at the minimum holding period. No other holding times were tested because in measurements of temperature distribution and flow rate on this plant, Burton (1958) showed that the sporicidal effect of increasing the holding time would be small in comparison with the effect of the heating-up period.

Operation of the plant was begun by re-circulating 10 gal of uninoculated milk. When the temperature of the heated milk in the plant was steady at the required value, inoculated milk was introduced, and the uninoculated milk diverted to waste. All the uninoculated milk having been diverted, a three-way cock controlling the flow along the by-pass was adjusted to allow treated inoculated milk to flow to both the sampling and diversion points. With the sampling cock closed, *c.* 5 gal of the inoculated milk were diverted to waste. Then, with milk still being diverted, the sampling cock was opened slowly to allow a controlled flow of milk to a 10 l. sterile aspirating jar connected aseptically to the plant. It was necessary to divert and

sample milk simultaneously because the jar could not deal with the full flow from the plant. A sample of the well-mixed inoculated raw milk was also taken as a control on the initial number of spores.

Treatment of samples

The treated milk was filled aseptically through a hooded outlet from the sampling jar into sterile flasks and tubes in 1×500, 5×100, 5×10, $5\times1{\cdot}0$, $5\times0{\cdot}1$ ml amounts and so on as required. Milk previously sterilized by heating to 135° in the plant and collected and dispensed aseptically was used as the dilution medium where necessary. The flasks and tubes of milk were incubated at 37° for 7 days.

Both colony and dilution counts were made on the control samples after steaming at 100° for 30 min to activate the spores. Colony counts were made on starch milk agar (SMA) (Grinsted & Clegg, 1955) and dilution counts in sterile UHT milk. In the later experiments, counts were also made in autoclaved litmus milk (ALM). Plates were incubated at 37° for 2 days and dilution tubes at 37° for 7 days.

As the test organism produced no marked visual change in UHT milk, all flasks and tubes of milk were examined microscopically. Doubtful positives were confirmed by streaking on SMA. The results of the dilution counts were obtained from McCrady's probability tables (Ministry of Health, 1956).

Results and Discussion

The various control counts on the raw milk (Table 1) show that there was a marked depression of the counts when the spores were incubated in UHT milk, these counts being sometimes one-hundredth of those observed in ALM. This suggests the possibility of a germination or post-germination growth inhibitor in the homogenized UHT milk.

Table 1. *The effect of different methods of estimation on the initial count of* Bacillus subtilis (786) *spores in raw milk, and of temperature on spore destruction in milk in a UHT plant*

Initial dilution count/ml in:		Initial colony count/ml	Treatment temp.	Final dilution count in milk	Destruction (% on SMA
UHT milk	Autoclaved litmus milk	SMA*	(°C)	(/ml)	count)
$1{\cdot}1\times10^5$	$6{\cdot}0\times10^6$	$7{\cdot}2\times10^6$	130·5	0·9	99·999988
$5{\cdot}0\times10^4$	$6{\cdot}0\times10^6$	$6{\cdot}9\times10^6$		0·6	99·999991
$<1{\cdot}0\times10^5$	$1{\cdot}7\times10^7$	$4{\cdot}8\times10^6$	132·0	0·2	99·999996
$2{\cdot}0\times10^5$	—	$4{\cdot}8\times10^6$		0·09	99·9999982
$<1{\cdot}0\times10^5$	—	$4{\cdot}7\times10^6$	133·5	0·001	99·99999998
$8{\cdot}0\times10^4$	$9{\cdot}0\times10^6$	$7{\cdot}3\times10^6$		0·001	99·99999998
$<1{\cdot}0\times10^5$	—	$4{\cdot}9\times10^6$	135·0	<0·0004	>99·999999992
$3{\cdot}5\times10^4$	$9{\cdot}0\times10^6$	$8{\cdot}2\times10^6$		<0·0004	>99·999999996

* Starch milk agar.

The results of the plant experiments given in the same table show that a 99·99999% destruction of spores was achieved at *c.* 130·5°. This temperature is lower than that necessary to give a similar degree of destruction of spores suspended in water (*c.* 135°)

(Williams *et al.* 1957). It is possible that the destruction of *B. subtilis* 786 spores in milk at 130·5° was less than the 99·99999% observed, but that an inhibitory effect exerted by the heated milk prevented the germination and growth of a proportion of the surviving spores. It seems desirable, therefore, that an operating temperature of 135° should be used with this plant to guarantee a destruction of 99·99999% of *B. subtilis* 786 spores.

Our thanks are due to Dr. A. T. R. Mattick for encouragement in this work and to Miss A. J. W. Harrison for technical assistance. We also wish to thank the A.P.V. Co., Ltd., Crawley, Sussex, for the loan of milk processing equipment, and the Director of the Microbiological Research Establishment, Porton, Wilts, for supplying the mass spore suspension.

References

Burton, H. (1958*a*). An analysis of the performance of an ultra-high-temperature milk sterilizing plant. I. Introduction and physical measurements. *J. Dairy Res.* **25**, 75.

Burton, H. (1958*b*). An analysis of the performance of an ultra-high-temperature milk sterilizing plant. II. Calculation of the bactericidal effectiveness. *J. Dairy Res.* **25**, 324.

Franklin, J. G., Williams, D. J. & Clegg, L. F. L. (1956). A survey of the number and types of aerobic mesophilic spores in milk before and after commercial sterilization. *J. appl. Bact.* **19**, 46.

Galesloot, Th. E. (1956). Een eenvoudige methode ter bepaling van het bacteriologisch effect van sterilisatie-processen voor melk toegepast op het steriliseren van melk in doorstroom -en flessensterilisatoren. *Ned. Melk-en Zuiveltijdschr.* **10**, 79.

Grinsted, E. & Clegg, L. F. L. (1955). Spore-forming organisms in commercial sterilized milk. *J. Dairy Res.* **22**, 178.

Ministry of Health (1956). *The Bacteriological Examination of Water Supplies. Rep. publ. Hlth med. Subj.* no 71. London: H.M.S.O.

Taylor, M. M. (1956). Sterilized milk as a growth medium for spore-forming bacilli. *Int. Dairy Congr., Rome*, **1**, 1.

Williams, D. J., Burton, H., Franklin, J. G. & Clegg, L. F. L. (1955). Some experiments with ultra-high-temperature pre-heating of sterilized milk. *J. Soc. Dairy Tech.* **8**, 192.

Williams, D. J., Franklin, J. G., Chapman, H. R. & Clegg, L. F. L. (1957). Methods of assessing the sporicidal efficiency of an ultra-high-temperature milk sterilizing plant. I. Experiments with suspensions of spores in water. *J. appl. Bact.* **20**, 43.

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METHODS OF ASSESSING THE SPORICIDAL EFFICIENCY OF AN ULTRA-HIGH-TEMPERATURE MILK STERILIZING PLANT. III. LABORATORY DETERMINATIONS OF THE HEAT RESISTANCE OF SPORES OF *BACILLUS SUBTILIS* IN WATER AND IN MILK

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SUMMARY: Thermal death curves for spores of *Bacillus subtilis* 786 have been determined in water and in milk. Generally a non-logarithmic order of death was observed. Numbers of survivors were lower in milk than in water, suggesting that there may be inhibitory factors in UHT sterilized milk which affect the germination and/or subsequent growth of heated spores.

The thermal death curves for spores suspended in milk yielded Q_{10} values of about 30 in the range 110–120°. This is higher than the figures previously reported in the literature for *B. subtilis* spores. Spores of a number of strains of *B. subtilis* were compared with strain 786 and all gave high Q_{10} values.

The results obtained in this work have been used to predict the destruction of spores at higher temperatures in a UHT plant (Burton *et al.* 1958). The calculated values agree well with the results obtained in the plant by Franklin *et al.* (1958).

Earlier papers in this series (Williams *et al.* 1957; Franklin *et al.* 1958) have dealt with the destruction of *Bacillus subtilis* spores in an ultra-high-temperature (UHT) milk sterilizing plant. It seemed desirable to investigate the effect of heat on spores by laboratory methods to assist in the interpretation of earlier results, and also to determine if it were possible to predict the sterilizing efficiency of a UHT milk sterilizing plant without resorting to the bacteriological experiments described in the previous papers.

Experimental Methods

Test organism

The spores used were those of a strain (786) of *Bacillus subtilis* originally isolated from bulk tanker milk (Franklin *et al.* 1956). Spore suspensions were prepared by the method described by Williams *et al.* (1957). Spores of this strain show marked heat activation, the germination of spores showing a tenfold increase after steaming at 100° for 30 min. Allowance for this was made when estimating the number of spores required in the inocula.

Determination of heat resistance

The technique for heat resistance tests was based on the method described by Stern & Proctor (1954). Glass capillary tubes (75×0·8 mm internal diameter) were cleaned thoroughly, sealed at one end and sterilized at 180° for 3 hr. A sterile micro-syringe (Agla) was used to fill 0·01 ml quantities of the spore suspension (*c.* 10^9/ml) into the

central portion of the capillaries. To avoid heating the spores the capillaries were carefully sealed in the pilot light of a Bunsen burner. For each time-temperature treatment to be investigated, ten capillaries (plus extras) held in a specially constructed rack were immersed in an electrically heated, thermo-regulated, stirred oil bath having a maximum variation of $\pm 0{\cdot}1°$.

After heating, the capillaries were cooled immediately by immersion in cold water, then washed under the tap, wiped with a clean cloth and placed in a test tube of acetone. After thorough washing in acetone the tubes were air dried, immersed in chromic acid for 5 min and washed in several changes of sterile distilled water. Finally 10 tubes were transferred aseptically to a 1 oz McCartney bottle containing 10 ml of sterile distilled water and a few glass beads. The capillaries were crushed by grinding with a sterile pipette and the resulting suspension was well mixed by shaking. The number of surviving spores in the suspension was estimated by colony and/or dilution counts. Ten unheated capillaries, as a control on the initial number of spores present, were treated in a similar way and the spore suspension 'heat-activated' (100° for 30 min) before plating.

Results

Experiments with spores in water

The first experiments were made with suspensions of *B. subtilis* 786 spores in water. The numbers of survivors were estimated by colony counts on starch milk agar (SMA)

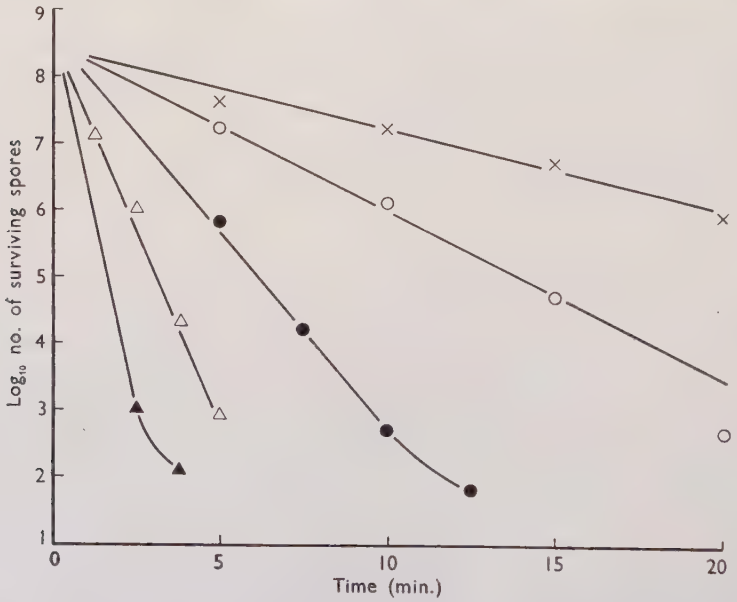

Fig. 1. The effect of temperature on the heat destruction of spores of *B. subtilis* 786 in water. Survivors determined by plating in starch milk agar.
Crosses, 110·0°; open circles, 112·5°; full circles, 115·0°; open triangles, 117·5°; full triangles, 120·0°.

(Grinsted & Clegg, 1955) after incubation at 37° for 2 days. The results were most unsatisfactory, showing a marked lack of reproducibility. This was not a result of the ageing of the spore suspension as the experiments proceeded, but appeared to be due to the recovery medium used.

Lengthy attempts to find a satisfactory culture medium for spores surviving a heat treatment produced a few ways of increasing the counts relative to those obtained in SMA, but these did not improve the reproducibility of results.

The results of one set of experiments in the range 110–120° are given in Fig. 1. These curves indicate a Q_{10} of *c.* 16 for the heat destruction of these spores in this temperature range. The value for such spores generally quoted is 8–10 (Rahn, 1945).

Experiments with spores in milk

As milk is the medium in which spores would be heated in practice and in which any survivors would have to germinate and grow, it was decided that more useful information might be obtained by heating spores in milk and using heated milk as a recovery medium.

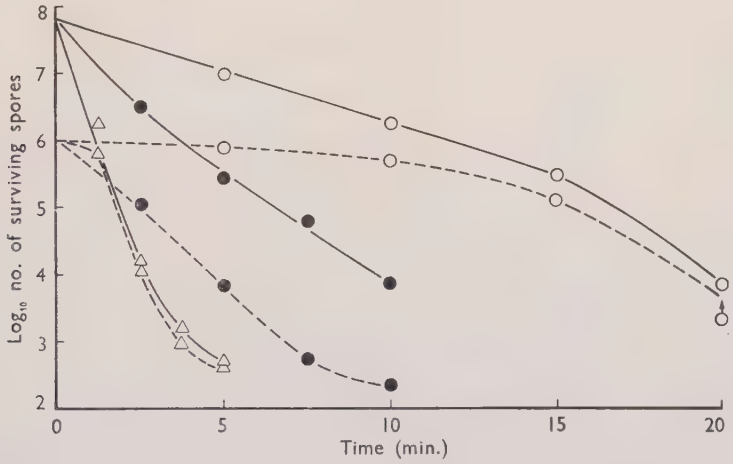

Fig. 2. The effect of temperature on the heat destruction of spores of *B. subtilis* 786 in milk. Survivors determined both by plating in starch milk agar and by dilution counts in UHT milk (series 1). Continuous lines, colony counts in starch milk agar; broken lines, dilution counts in UHT milk; open circles, 112·5°; full circles, 115·0°; open triangles, 117·5°.

The above work was therefore repeated, using spores suspended in UHT milk which had been previously sterilized in the plant. The spores were heated as before at temperatures of 112·5°, 115·0° and 117·5° (series 1). The survivors were estimated by dilution counts in the UHT milk (incubated 7 days at 37°) and by colony counts on SMA (incubated 2 days at 37°). The results are given in Fig. 2. These curves give a Q_{10} of *c.* 30, which is much higher than the value of 16 determined for spores

heated in water. It was thought that the survivor curves might possibly have been affected by coagulation of milk on heating, resulting in clumping of spores. Accordingly some of the capillaries were examined microscopically, but no evidence of this was found. Moreover the dilution counts are lower than the colony counts, suggesting some inhibitory action of the UHT milk on germination and/or subsequent growth in a proportion of the survivors. A similar inhibitory effect was also reported by Franklin *et al.* (1958). Consequently it was decided to test another batch of UHT sterilized milk for inhibitory properties before proceeding further.

Spores of *B. subtilis* 786 were heated in the UHT milk, and also in sterile homogenized and sterile skim milk, to determine whether the inhibitory effect, if present, was related to the presence or the dispersion of fat. The homogenized and skim milks were sterilized by autoclaving at 121° for 20 min. Surviving spores were estimated by dilution counts in the above three types of milk, and also by colony counts in SMA. Heat treatments of 100°, 110° and 117·5° for 5 min were investigated. The results (Table 1) show no real difference between the three milks, tested either as heating or as recovery media. The dilution counts were generally slightly lower than the colony counts.

Table 1. *The effect of different types of milk used both as heating and recovery media on the destruction of* B. subtilis *spores*

Heating medium	*Treatment temp. (°C)	Survivor counts (/ml) in different recovery media			
		Dilution counts in:			Colony counts in starch milk agar
		UHT whole milk	Autoclaved whole homogenized milk	Autoclaved skim milk	
UHT whole milk	100	$1{\cdot}7\times10^{8}$	$3{\cdot}5\times10^{7}$	$7{\cdot}0\times10^{7}$	$1{\cdot}9\times10^{8}$
	110	$9{\cdot}0\times10^{7}$	$3{\cdot}5\times10^{7}$	$1{\cdot}3\times10^{7}$	$2{\cdot}2\times10^{8}$
	117·5	$1{\cdot}4\times10^{3}$	$3{\cdot}0\times10^{3}$	$2{\cdot}5\times10^{3}$	$5{\cdot}3\times10^{3}$
Autoclaved whole homogenized milk	100	$1{\cdot}4\times10^{8}$	$9{\cdot}0\times10^{7}$	$3{\cdot}5\times10^{7}$	$2{\cdot}4\times10^{8}$
	110	$9{\cdot}0\times10^{7}$	$3{\cdot}5\times10^{7}$	$1{\cdot}7\times10^{7}$	$3{\cdot}0\times10^{8}$
	117·5	$6{\cdot}0\times10^{2}$	$3{\cdot}5\times10^{2}$	$6{\cdot}0\times10^{2}$	$2{\cdot}7\times10^{3}$
Autoclaved skim milk	100	$1{\cdot}7\times10^{8}$	$3{\cdot}0\times10^{7}$	$9{\cdot}0\times10^{7}$	$2{\cdot}6\times10^{8}$
	110	$9{\cdot}0\times10^{7}$	$1{\cdot}7\times10^{7}$	$6{\cdot}0\times10^{7}$	$2{\cdot}6\times10^{8}$
	117·5	$6{\cdot}0\times10^{2}$	$3{\cdot}5\times10^{3}$	$9{\cdot}0\times10^{2}$	$4{\cdot}7\times10^{3}$

* The time of treatment was 5 min in all cases.

It was then decided to repeat the previous heat resistance tests using this second batch of UHT milk, in which there was no indication of any inhibitory effect, as both the heating medium and recovery medium for the dilution counts (series 2). The results (Fig. 3) again yielded a high Q_{10} value of *c.* 30. This time colony and dilution counts showed closer agreement, there being no evidence of any inhibitory effect in the recovery medium. The unexpectedly high Q_{10} values obtained suggested that *B. subtilis* 786 spores might be atypical and so the work was repeated with the spores of four other strains of *B. subtilis* in addition to strain 786. Heating was carried out in the non-inhibitory batch of UHT milk and the survivors estimated by plating on SMA (series 3). The results of this experiment are given in Fig. 4, (a)–(e). The

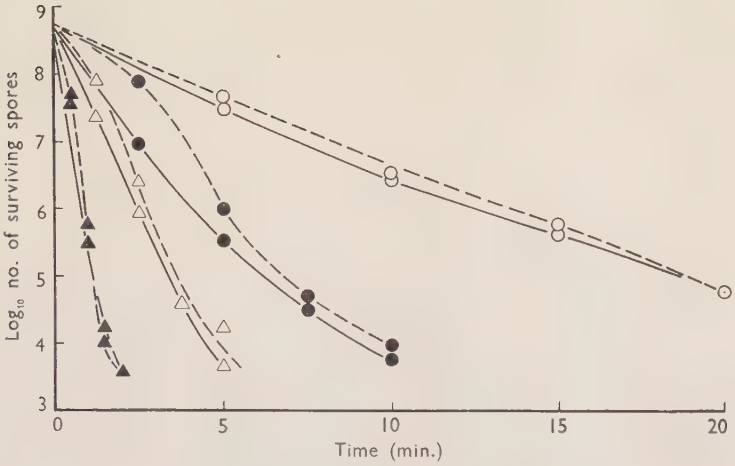

Fig. 3. The effect of temperature on the heat destruction of spores of *B. subtilis* 786 in milk. Survivors determined both by plating in starch milk agar and by dilution counts in UHT milk (series 2). Continuous lines, colony counts in starch milk agar; broken lines, dilution counts in UHT milk; open circles, 112·5°; full circles, 115·0°; open triangles, 117·5°; full triangles, 120·0°.

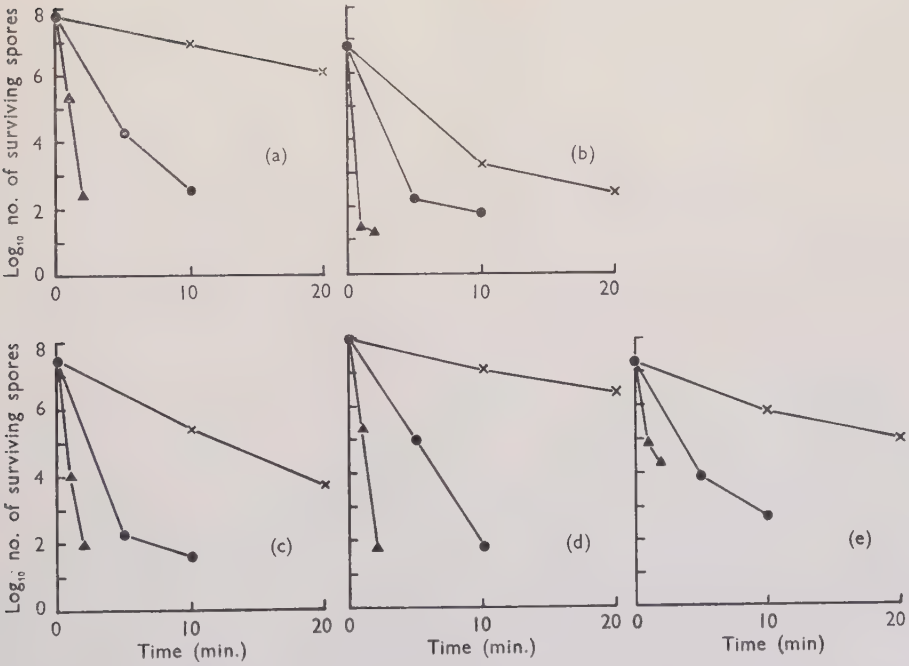

Fig. 4. The effect of temperature on the heat destruction of five strains of *B. subtilis* in milk. Survivors determined by plating in starch milk agar (series 3).
B. subtilis strains: (a), 786; (b), 19; (c), 17; (d), 25; (e), 115. Crosses, 110°; circles, 115°; triangles, 120°.

sources of the 5 strains tested, and their Q_{10} values, are given in Table 2. Although these values have each been determined on limited data, it is evident that the Q_{10} values of all five strains were high and none differed greatly from the value initially obtained for strain 786.

Table 2. *The sources and Q_{10} values for heat destruction of spores of five strains of* B. subtilis

Strain no.	Source	Q_{10} value
17	Raw milk	19
115	Italian sterilized milk	20
19	NCIB* 8159	25
786	Raw bulk tanker milk	32
25	Dust	35

* National Collection of Industrial Bacteria.

Discussion

Several explanations of the high Q_{10} values observed in this work are possible. First, the spores may have been physically more susceptible to heat when suspended in UHT milk. This seems unlikely. Second, toxic substances may have been present in UHT milk which would enhance the lethal effect of the heat treatments. Third, an inhibitory agent, which could affect germination and/or subsequent growth of the heated spores, may have been present during heating.

There appear to be two inhibitory effects in UHT milk, one associated with the heating medium, resulting in high Q_{10} values, and the other with the recovery medium used in the dilution counts. The inhibitory action of UHT milk is being investigated.

One of the interesting features of this work is the evidence in Figs. 3 and 4 and to a lesser extent in Figs. 1 and 2 that the heat destruction curves of *B. subtilis* spores are sigmoid, not exponential as claimed by a number of workers, e.g. Rahn (1945). Vas & Proszt (1957) have recently discussed the arguments for and against a logarithmic order of death. The matter is important in this work and has been taken into account by Burton *et al.* (1958) in using these data to predict the destruction of spores at higher temperatures.

From the results of series 1–3 reported here Burton *et al.* (1958) have calculated the Q_{10} value over the range 112·5°–120° for *B. subtilis* 786 spores heated in milk and found it to be 31·7. The results were used to construct a theoretical curve by the method described by Burton (1958*a*,*b*) to predict the behaviour of spores heated at temperatures of *c.* 135°, i.e. the temperature recommended previously for sterilizing milk in the APV HX-heater (Franklin *et al.* 1958). This curve is shown in Fig. 5, together with the curve from results obtained in practice with the plant (Franklin *et al.* 1958). The two curves can be seen to agree closely. Therefore, providing the thermal death characteristics of the spores concerned are known, it seems possible to predict by the theoretical method of Burton (1958*a*,*b*) how spores will behave in a UHT milk sterilizing plant without resorting to bacteriological experiments with the plant itself.

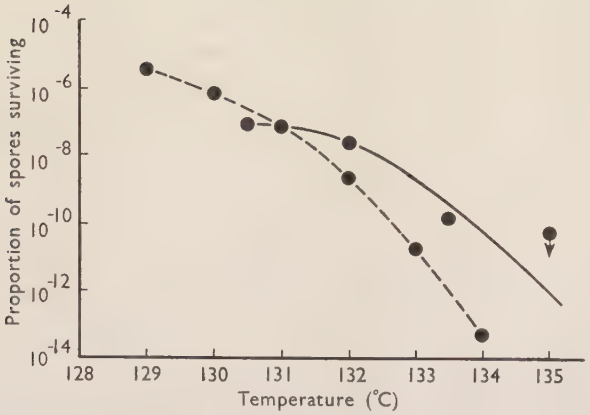

Fig. 5. A comparison of actual and calculated thermal destruction curves for spores of *B. subtilis* 786 suspended in milk in an APV ultra-high-temperature plate heater.
Continuous line, experimental results; broken line, calculated values.

Grateful acknowledgement is made to Mr. Burton for the calculations on the data used for Fig. 5 and for most useful discussion; also to Dr. A. T. R. Mattick for helpful advice during the course of this work.

References

Burton, H. (1958*a*). An analysis of the performance of an ultra-high-temperature milk sterilizing plant. I. Introduction and physical measurements. *J. Dairy Res.* **25**, 75.

Burton, H. (1958*b*). An analysis of the performance of an ultra-high-temperature milk sterilizing plant. II. Calculation of bactericidal effectiveness. *J. Dairy Res.* **25**, 324.

Burton, H., Franklin, J. G., Williams, D. J., Chapman, H. R., Harrison, A. J. W. & Clegg, L. F. L. (1958). An analysis of the performance of an ultra-high-temperature milk sterilizing plant. III. Comparison of experimental and calculated sporicidal effects for a strain of *Bacillus subtilis*. *J. Dairy Res.* **25**, 338.

Franklin, J. G., Williams, D. J. & Clegg, L. F. L. (1956). A survey of the number and types of aerobic mesophilic spores in milk before and after commercial sterilization. *J. appl. Bact.* **19**, 46.

Franklin, J. G., Williams, D. J., Chapman, H. R. & Clegg, L. F. L. (1958). Methods of assessing the germicidal efficiency of an ultra-high-temperature milk sterilizing plant. II. Experiments with suspensions of spores in milk. *J. appl. Bact.* **21**, 47.

Grinsted, E. & Clegg, L. F. L. (1955). Spore-forming organisms in commercial sterilized milk. *J. Dairy Res.* **22**, 178.

Rahn, O. (1945). Physical methods of sterilization of micro-organisms. *Bact. Rev.* **9**, 1.

Stern, J. A. & Proctor, B. E. (1954). A micro-method and apparatus for the multiple determination of rates of destruction of bacteria and bacterial spores subjected to heat. *Food Technol., Champaign*, **8**, 139.

Vas, K. & Proszt, G. (1957). Observations on the heat destruction of *Bacillus cereus*. *J. appl. Bact.* **20**, 431.

Williams, D. J., Franklin, J. G., Chapman, H. R. & Clegg, L. F. L. (1957). Methods of assessing the sporicidal efficiency of an ultra-high-temperature milk sterilizing plant. I. Experiments with suspensions of spores in water. *J. appl. Bact.* **20**, 43.

(*Received* 13 *December*, 1957)

Thomas, S. B., Hobson, P. M. & Elson, K. (1958). *J. appl. Bact.* **21** (1), 58–64.

COLI-AEROGENES BACTERIA FROM FARM DAIRY EQUIPMENT AND RAW MILK

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SUMMARY: A series of 1,040 cultures of coli-aerogenes bacteria isolated at 30° from farm dairy equipment and raw milk was classified according to the recommendations of the Coliform Sub-Committee of the Society for Applied Bacteriology (Report, 1949). The distribution of species and types isolated from milk did not differ materially from that for dairy equipment, *Klebsiella cloacae*, *K. aerogenes* and *Citrobacter freundii* being the dominant species, while the incidence of *Escherichia coli* was relatively low. There was no marked seasonal incidence of any type, though *E. coli* I formed a slightly higher proportion of the coli-aerogenes microflora in winter, whereas *K. aerogenes* I was slightly higher in summer. Many of the cultures of the dominant types, *K. cloacae*, *K. aerogenes* I and *Cit. freundii* I, did not form acid and gas from lactose in two days at 37°. Consequently 37° negative, anaerogenic and paracolon strains constituted a relatively high proporton (49%) of the coli-aerogenes microflora.

The findings of thirteen previous investigators on the incidence of different types of coli-aerogenes bacteria isolated at 37° from raw milk were reviewed by Thomas (1955), who also gave some preliminary data for the distribution of the types isolated at 30° from farm milk supplies. The present report deals with the classification of the coli-aerogenes bacteria isolated at 30° from rinses of farm dairy equipment examined within 6 hr of sampling and from samples of 10–18 hr old raw milk from farms.

Experimental Methods

Sampling

Rinses of farm dairy equipment were taken at regular fortnightly intervals during 1951–56 according to the technique recommended by the Ministry of Agriculture (1946), except that the 500 ml rinse solution was poured twice over the equipment, 0·05% sodium thiosulphate was incorporated in the rinse solutions (quarter-strength Ringer) used for rinsing hypochlorite treated equipment, and 5 ml of a 4% lecithin-6% Lubrol W solution were added to the rinse before rinsing equipment which had been washed in detergent-quaternary ammonium compound solutions. The rinses were examined within 6 hr of sampling by inoculating 1 ml of rinse and 1 ml of decimal dilutions into MacConkey's broth incubated for 5 days at 30°.

Three-ounce samples of morning and evening milk, taken from single cans at the farm stand, were held at atmospheric shade temperature until examination, when 10–18 hr old. During 1951–53, decimal dilutions of the milk were prepared up to 10^{-4} and 1 ml quantities of each dilution inoculated into MacConkey's broth, which

was incubated for 5 days at 30°, while during 1954–56 1 ml quantities of each dilution were inoculated directly into violet red bile agar (VRB) which was incubated for 20–24 hr at 30°.

Isolation and differential tests

Streaks on Levine's eosin methylene blue agar were made from the highest positive dilution of MacConkey's broth and, after incubation for 1 or 2 days at 30°, one to three well isolated colonies having the characteristic appearance of coli-aerogenes bacteria were picked into peptone water. Up to six well isolated coli-aerogenes-like colonies were picked from the VRB agar into peptone water.

A 24 hr peptone water culture at 30° provided the inoculum for the differential tests recommended in Report (1949), an incubation temperature of 30° being used for the IMViC tests. Non-lactose-fermenters are defined as 37° negative when no gas was formed from lactose in 2 days at this temperature, anaerogenic when acid but no gas was formed in 5 days at 30° and paracolon when neither acid nor gas was formed at 30°. The nomenclature proposed in Report (1956) was applied to the classification of the strains studied.

Results and Discussion

Classification

Duplicate cultures from the same rinse or milk sample showing identical reactions in all media were discarded, leaving 429 classifiable cultures from farm dairy equipment and 611 from raw milk. The incidence of different types in these 1,040 cultures is summarized in Table 1.

Klebsiella constituted the dominant genus, with *K. cloacae* the most common species in the coli-aerogenes microflora of both dairy equipment and milk. *Cit. freundii* was relatively abundant, forming 20% of the cultures from the former and 30% of those from the latter. The incidence of *E. coli* I was relatively low.

The distribution of different types isolated from milk did not differ materially from that for dairy equipment, the difference being never greater than 6%. *E. coli* I was slightly more abundant on equipment, whereas *Cit. freundii* I was more abundant in milk. Non-lactose-fermenters (37° negative, anaerogenic or paracolon strains) were more abundant among the cultures isolated from dairy equipment (55% of 429) than those from milk (45% of 611). This difference was most marked with *K. aerogenes* I (64% from equipment and 37% from milk) and *K. cloacae* (68% from equipment and 58% from milk). This difference could not be attributed to the use of VRB agar for the primary isolation of milk cultures during the latter half of the investigation, as a similar trend has been observed in cultures isolated by means of MacConkey's broth (Thomas, 1953).

Variations in other characteristics were observed in most species. Among the most striking were: the failure of 14 (12%) of the *E. coli* I cultures to form gas at 44°; the ability of 22 (37%) of the *Cit. freundii* II cultures to do so; liquefaction of gelatin within 21 days at 22° by 59 (27%) of the *Cit. freundii* I cultures, and failure to

Table 1. *The classification of* 1,040 *cultures of coli-aerogenes bacteria isolated at* 30° *from farm dairy equipment and raw milk*

Organism and biotype	Cultures isolated from					
	Dairy equipment			Raw milk		
	No.		%	No.		%
Escherichia coli I	56			44		
44° negative	1			5		
37° negative	1	62	14·5	0	52	8·5
anaerogenic	3			1		
paracolon	1			2		
E. coli II	4			17		
44° positive	1			2		
37° negative	8			12		
37° negative, gelatin positive	2			0		
anaerogenic	4	22	5·1	12	51	8·3
anaerogenic, gelatin positive	2			0		
paracolon	0			8		
paracolon, gelatin positive	1			0		
Citrobacter freundii I	26			39		
44° positive	2			0		
gelatin positive	4			22		
37° negative	27			30		
37° negative, gelatin positive	6	75	17·5	10	144	23·6
anaerogenic	4			6		
anaerogenic, gelatin positive	1			5		
paracolon	2			24		
paracolon, gelatin positive	3			8		
Cit. freundii II	6			20		
44° positive	4			18		
gelatin positive	0			4		
37° negative	2	12	2·8	1	47	7·7
37° negative, gelatin positive	0			2		
anaerogenic	0			1		
paracolon	0			1		
Klebsiella aerogenes I	37			67		
44° positive	0			1		
citrate negative	1			6		
37° negative	59			20		
37° negative, citrate negative	4	106	24·7	2	118	19·3
anaerogenic	0			4		
anaerogenic, citrate negative	0			1		
paracolon	5			10		
paracolon, citrate negative	0			7		
K. cloacae	46			70		
indole positive	0			3		
citrate negative	0			1		
37° negative	61			37		
37° negative, citrate negative	0	145	33·8	1	176	28·8
anaerogenic	3			18		
paracolon	35			43		
paracolon, citrate negative	0			3		
K. aerogenes II	3			13		
44° positive	0			1		
gelatin positive	1			2		
37° negative	2	7	1·6	4	23	3·8
37° negative, gelatin positive	1			1		
paracolon	0			2		
Total cultures		429			611	

utilize citrate by 21 (9%) of the *K. aerogenes* I cultures. We did not study the persistence of such variations beyond observing that they were generally unchanged on re-checking within a few weeks.

Seasonal incidence of different types

The results summarized in Table 2 show that there was no marked seasonal incidence of any species. *E. coli* I formed a slightly higher proportion of the coli-aerogenes flora of both dairy equipment and milk during winter (November–April) than during summer (May–October), but the difference was very much less than that reported by Malcolm (1933), who found that *E. coli* constituted 71% of the cultures isolated at 37° from milk in winter compared with 54% in summer. The results resemble more closely those obtained by Kampe (1953), who showed that *E. coli* formed about the same proportion of the cultures isolated from ordinary milk in winter as in summer.

The proportional incidence of *K. aerogenes* I was slightly higher for dairy equipment and more so in milk, during summer. It is probable that *Klebsiella* and *Citrobacter* multiply as well, if not better, than *Escherichia* in the milk deposits of unsterile milking utensils at summer temperatures. Malling Olsen (1952) has shown that the growth of *K. aerogenes* was more than ten times that of *E. coli* I in raw aseptically drawn milk in 24 hr at 15°.

Table 2. *The percentage seasonal incidence of different types of coli-aerogenes bacteria isolated from farm dairy equipment and raw milk*

Organism and type	Farm dairy equipment		Farm milk supplies	
	Winter	Summer	Winter	Summer
Escherichia coli I	16·1	12·9	9·8	7·2
E. coli II	4·3	5·8	8·2	8·5
Citrobacter freundii I	16·6	18·3	25·8	21·3
Cit. freundii II	2·0	3·5	7·2	8·2
Klebsiella aerogenes I	22·9	26·6	15·4	23·3
K. cloacae	37·6	30·3	31·0	26·6
K. aerogenes II	0·5	2·6	2·6	4·9
Total cultures	(205)	(224)	(306)	(305)

Cultures from rubber and metal surfaces

A comparison of the incidence of different types isolated from rubber surfaces (teat cup liners and milk tubes of milking machines) and from metal surfaces (milking pails, receiving pans, coolers and strainers) is given in Table 3. *E. coli* I formed a higher proportion of the coli-aerogenes flora of rubber surfaces than of metal surfaces, while *K. aerogenes* I and *K. cloacae* were correspondingly more frequent on metal surfaces than on rubber. These results suggest that *E. coli* I may survive better than *Citrobacter* and *Klebsiella* in the fatty deposits in the pores of milking machine rubbers during cleansing treatments, since it is not conceivable that it should multiply more actively than *Citrobacter* and *Klebsiella* in this habitat.

Table 3. *The classification of coli-aerogenes bacteria isolated from rubber and metal surfaces of farm dairy equipment*

Organism and type	Rubber surfaces		Metal surfaces	
	No.	%	No.	%
Escherichia coli I	39	21·1	13	10·2
E. coli II	7	3·8	9	7·1
Citrobacter freundii I	33	17·9	18	14·2
Cit. freundii II	5	2·7	5	3·9
Klebsiella aerogenes I	46	25·0	37	29·1
K. cloacae	50	27·2	43	33·9
K. aerogenes II	4	2·2	2	1·6
Total cultures	184		127	

Classification of cultures forming or not forming gas from lactose

As there were no marked differences in the incidence of different species and types isolated from dairy equipment and from milk, the cultures from both sources have been combined for the following summaries. The classification of the 37° positive cultures forming acid and gas from lactose in 2 days at 37°, the cultures negative at 37° but forming gas from lactose in 5 days at 30°, the anaerogenic cultures forming acid but no gas from lactose in 5 days at 30° and the paracolon cultures failing to form acid or gas in 5 days at 30°, is summarized in Table 4.

Cultures positive at 37°. *Klebsiella* constituted 48%, *Citrobacter* 27% and *Escherichia* 25% of the 527 cultures of this group. *K. cloacae*, *K. aerogenes* I and *E. coli* I were the dominant types, with *Cit. freundii* I quite frequent. Most previous investigators have reported *E. coli* I to constitute from about 40% to 55% of coli-aerogenes cultures isolated at 37° from milk, but it only formed 20% of our 37° positive cultures. It is thus evident that isolation at 37° does not give a true picture of the distribution even of the 37° positive types.

Cultures negative at 37°. *Klebsiella* constituted 66%, *Citrobacter* 26% and *Escherichia* 8% of the 293 cultures of this group. The dominant types in order of frequency were *K. cloacae*, *K. aerogenes* I and *Cit. freundii* I, while 37° negative strains of *E. coli* II were fairly frequent.

Table 4. *The classification of the* 37° *positive,* 37° *negative, anaerogenic and paracolon cultures of the coli-aerogenes bacteria isolated at* 30°

Organism and type	37° positive		37° negative		Total 30° positive		Anaerogenic and paracolon		Total classifiable	
	No.	%	No.	%	No.	%	No.	%	No.	%
E. coli I	106	20·1	1	0·3	107	13·0	7	3·2	114	10·9
E. coli II	24	4·5	22	7·5	46	5·6	27	12·3	73	7·0
Cit. freundii I	93	17·6	73	24·9	166	20·2	53	24·9	219	21·1
Cit. freundii II	52	9·9	5	1·7	57	6·9	2	0·9	59	5·7
K. aerogenes I	112	21·3	85	29·0	197	24·2	27	12·3	224	21·5
K. cloacae	120	22·8	99	33·8	219	26·7	102	45·5	321	30·9
K. aerogenes II	20	3·8	8	2·8	28	3·4	2	0·9	30	2·9
Total cultures	527		293		820		220		1,040	

Cultures positive at 30°. The distribution of different types among the 810 cultures which formed acid and gas from lactose in 5 days at 30°, i.e. the combined 37° positive and 37° negative cultures, was very similar to that among the total 1,040 cultures, *K. cloacae* and *K. aerogenes* I being the most common types, closely followed by *Cit. freundii* I.

Anaerogenic and paracolon cultures. K. cloacae was markedly dominant among the 220 cultures of this group, forming almost half the strains, while *Cit. freundii* I constituted another 25% and *E. coli* II and *K. aerogenes* I each formed 12%.

Distribution of temperature groups

The distribution of the three temperature groups among different types is summarized in Table 5. The majority of the cultures of *E. coli* I were 37° positive, but a few failed to form gas even in 5 days at 30°. On the other hand the cultures of *E. coli* II were about equally divided among the three temperature groups, a surprisingly high proportion being anaerogenic or paracolon.

A third of the cultures of *Cit. freundii* I were 37° negative and another quarter anaerogenic or paracolon, but most of the cultures of *Cit. freundii* II were 37° positive.

K. aerogenes I contained a high proportion of 37° negative cultures, though anaerogenic or paracolon strains were not very common. The incidence of both 37° negative and anaerogenic or paracolon strains was exceptionally high in *K. cloacae*, and as this was the dominant species in the coli-aerogenes flora, forming close on a third of the 1,040 cultures studied, it contributed a high proportion of the strains which did not form gas from lactose at 37°. A relatively high proportion of the few cultures of *K. aerogenes* II was 37° positive.

Table 5. *The distribution of temperature groups among different types of coli-aerogenes bacteria isolated from dairy equipment and raw milk*

Organism and type	Temperature group						Total cultures
	37° positive		37° negative		Anaerogenic and paracolon at 30°		
	No.	%	No.	%	No.	%	
E. coli I	106	93·0	1	0·9	7	6·1	114
E. coli II	24	32·9	22	30·1	27	37·0	73
Cit. freundii I	93	42·5	73	33·3	53	24·2	219
Cit. freundii II	52	88·1	5	8·5	2	3·4	59
K. aerogenes I	112	50·0	85	37·9	27	12·1	224
K. cloacae	120	37·4	99	30·8	102	31·8	321
K. aerogenes II	20	66·7	8	26·7	2	6·6	30
Total cultures	527	50·7	293	28·2	220	21·1	1,040

Conclusion

The futility of attempting to study the distribution of different types of coli-aerogenes bacteria on dairy equipment and in milk by isolation at 37° is illustrated by the occurrence of 513 cultures which failed to form gas from lactose in 2 days at 37°

among the 1,040 studied by isolation at 30° during the present investigation, i.e. close on 50%.

Characteristic purple-red colonies over 0·5 mm in diameter developing on violet red bile agar in 20–24 hr at 30° included anaerogenic and paracolon strains as well as 37° positive and 37° negative strains. Thus, among 394 colonies derived from 79 milk samples, 37% were 37° negative and a further 11% anaerogenic or paracolon.

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References

Kampe, A. (1953). On coliform bacteria in raw milk. *Int. Dairy Congr. The Hague* **2**, 148.

Malcolm, J. F. (1933). The occurrence of coliform bacteria in milk. *J. Dairy Res.* **5**, 15.

Malling Olsen, E. (1952). *On Coliform Bacteria in Milk.* Copenhagen: Kandrup & Wunsch.

Ministry of Agriculture (1946). *Examination of washed milk churns.* Technique no. C 168/TPY. London: H.M.S.O.

Report (1949). The classification of the coli-aerogenes bacteria. Report of the Coliform Sub-Committee. *Proc. Soc. appl. Bact.* (2), 3.

Report (1956). The nomenclature of coli-aerogenes bacteria. *J. appl. Bact.* **19**, 108.

Thomas, S. B. (1953). Unpublished data.

Thomas, S. B. (1955). Coli-aerogenes bacteria in raw milk. *J. appl. Bact.* **18**, 331.

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Billing, E. & Cuthbert, W. A. (1958). *J. appl. Bact.* **21** (1), 65–78.

'BITTY' CREAM: THE OCCURRENCE AND SIGNIFICANCE OF *BACILLUS CEREUS* SPORES IN RAW MILK SUPPLIES

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SUMMARY: Methods for determining the *Bacillus cereus* content of milks and of rinses of dairy equipment are described and their limitations discussed. Milk samples from various sources were examined throughout the year for 'bittiness' as well as for *B. cereus*. The organism was not detected in all samples showing bittiness. There was a marked seasonal variation in the *B. cereus* index of raw milk supplies; maximum numbers were obtained from July to September and minimum numbers in April and May. Rinses of farm dairy equipment yielded few *B. cereus* spores but milk cans not uncommonly contained large numbers, especially in the summer months, when 10·5% of cans showed more than 5×10^3/can. Preliminary observations on other sources of *B. cereus* are described and some of the problems of the control of this organism in raw milk supplies are discussed.

The problem of 'bitty' cream was investigated in detail by Stone & Rowlands (1952) and Stone (1952*a*,*b*). They showed that the fault was associated with *Bacillus cereus* and *B. mycoides* and that, once milk had become infected, the rate of development of the fault depended almost entirely on the temperature of storage. As it appeared that infection of milk supplies with these organisms was inevitable, the only satisfactory method of controlling 'bittiness' was, in their opinion, to keep the milk as cool as possible after pasteurization.

In spite of these findings, some dairies, in an attempt to reduce the incidence of the fault, have threatened to reject milk on the ground that it contains *B. cereus* spores, and this investigation was started as a result of such action at two local dairies.

When asked for advice by farmers in these circumstances, we were handicapped by having no satisfactory method of checking that such a farm was in fact consistently supplying milk with an abnormally high content of *B. cereus* spores, or of examining dairy equipment and other possible sources for this organism. Our first object therefore was to devise techniques whereby we could obtain an estimate of the numbers of *B. cereus* spores and vegetative cells in milk samples and in dairy equipment and can rinses. Our second object was, by using these techniques, to determine the *B. cereus* spore content of milk supplies from a variety of sources and also to obtain some information about the origin of these spores and whether control measures were practicable at the farm.

Materials and Methods

Egg yolk media. A 10% (w/v) suspension of egg yolk was prepared according to the method of McGaughey & Chu (1948). For use, 1 ml of the suspension was added aseptically to 10 ml of medium. Later a simplified method of preparing egg yolk suspension was devised (Billing & Luckhurst, 1957).

Counting methods

Dilution method. This was used for nearly all the milk samples examined and for the majority of milk can rinses. For milks, about 60 ml of each sample were transferred aseptically to a 10×1 in. test tube which was closed with a 'Suba-Seal' (Williams Freeman & Co., Ltd., Suba-Seal Works, Peel Street, Barnsley). Soiling of the top 2 in. of the tube was avoided. The stoppers were secured with rubber bands and the tubes were completely immersed in a water bath at 63·5° (±1°) for 30 min after the temperature in a control tube had reached 62·5°. The tubes were cooled in iced water, inverted 3 times and the milk aseptically distributed as follows: 5×10 ml in sterile boiling tubes or MacCartney bottles, 5×1 ml in 5 ml of litmus milk, and 5×1 ml of a 10^{-1} dilution in 5 ml of litmus milk.

The sets of tubes were incubated at 22° (±1°). After 24 and 48 hr (±2 hr) the 10 ml quantities of milk were examined for evidence of bittiness or coagulation by gently swirling 25 times. After 72 hr (±2 hr), before disturbing the milk in any of the tubes, changes which had taken place in the litmus-containing milks were recorded (Plate 1a) and a loopful of cream from the surface of each of the 15 tubes inoculated on to a dry egg yolk agar plate which was incubated at 22°. Final changes in the 10 ml tubes were then recorded.

The plates were examined after 24 hr (±2 hr) for evidence of lecithinase activity (Plate 1b). Spots which were surrounded by a well defined zone of opacity typical of *B. cereus* were recorded as positive. *B. cereus* var. *mycoides*, because of its spreading growth, did not always produce marked opacity, but where this growth was observed the result was recorded as positive. It can be seen from Plate 1a that the milk tubes which gave no evidence of change did not yield colonies typical of *B. cereus* in the confirmatory egg yolk medium (Plate 1b). The probable number of *B. cereus* organisms (including *B. cereus* var. *mycoides*)/100 ml of milk was determined using McCrady's tables.

In devising this technique, several practical points arose. Apart from the inherent inaccuracies of a dilution technique, it was realized that such a method would not necessarily give a complete picture of the *B. cereus* content of the milk. When tubes were incubated longer, higher counts were sometimes obtained, presumably due to delayed spore germination: but when incubation was continued beyond 5 days anaerobic bacteria became active and the *B. cereus* counts often showed a decrease. Spores germinating after 2 days at 22° would not be of practical importance in pasteurized milk and 3 days incubation was considered reasonable and convenient. The count so obtained/100 ml is referred to as the *B. cereus* index. It is presumptive, and includes *B. cereus* var. *mycoides*.

It was observed in early experiments that different strains of *B. cereus* varied in the intensity of the lecithinase reaction. Some strains produce a stronger reaction at 37°, others at 22°. As lecithinase activity at lower temperatures was of more practical importance, 22° was chosen for incubating both milks and plates. The ability of *B. cereus* to grow rapidly at 22° meant that normally a well defined zone of opacity on egg yolk plates was discernable after 24 hr and sometimes within a few hours of inoculation.

False positive reactions were not common, and with experience could readily be

distinguished from the typical *B. cereus* reaction. In such atypical reactions the zones were generally smaller and either denser with a more sharply defined edge or more granular than those of *B. cereus*. The organisms causing these false positive reactions were normally either *Pseudomonas* or *Achromobacter* species. A higher heat treatment temperature (e.g. 80° for 10 min) might have destroyed these organisms, but it was felt that the test would be of more value if it reflected practical conditions. That this is so is shown by the results of Clegg *et al.* (1956), who found that heating to 80–90° for 30 min retarded bittiness and that *B. cereus* spores were completely destroyed at temperatures above 90° for 30 min.

As the numbers of *B. cereus* were frequently of the order of a few cells/100 ml of milk or rinse, transfer of the material after heat treatment might be criticized because of the risk of contamination. Blank tests however have never shown positive results and the risk is not thought to be great.

For rinses, etc., where the numbers of *B. cereus* cells were expected to be higher than in milk, the techniques followed a similar pattern except that a 10^{-2} dilution was used and the 10 ml quantities omitted. In these cases the tubes were laboratory pasteurized after inoculation.

Surface plate method. This method was sometimes used for the examination of rinses. Egg yolk agar plates were thoroughly dried by incubating them closed overnight and inverted and open for 4 hr next day at 37°; where a 56° incubator was available, a 1 hr incubation with the plates inverted and open was sufficient. A 0·5 ml quantity of the material under test was delivered on to the agar while it was still warm, spread over as much of the surface as possible by tilting, and the plate left on a level surface. This quantity was absorbed in 10–15 min with a properly dried plate. Plates were examined after 24 and 48 hr (±2 hr) at 22° for the presence of colonies giving a typical positive reaction. *B. cereus* colonies normally gave this in 24 hr but a further examination at 48 hr was made, for late developers. By this time, especially with unheated material, other organisms giving a weaker reaction were often present but could readily be distinguished both by the type of reaction and the colony form.

This method had the advantage that results were available in 24–48 hr but could only be applied when counts were comparatively high. It was also used occasionally in the case of pasteurized milks which were known to have high counts. The reaction was obscured if undiluted milk were plated, but a 10^{-1} dilution was satisfactory.

Samples

Sampling of milks commenced in January 1955 at two laboratories in the North of England. In July 1955 a third laboratory, in the South, commenced sampling. Initially a single screening test was made on about 100 TT milk supplies and a number of farms was selected from this group in the hope of obtaining a variety of supplies showing a wide range of bacteriological quality. Subsequent results showed that this was achieved; of the samples tested at the 3 laboratories over a 12 month period the percentage giving a colony count of over 10^4/ml varied from 0–100%. Most of the work described was done on morning samples taken at a dairy near the laboratory and tested as soon as possible. This meant that the milk was

seldom more than 6 hr old. Evening samples taken at the same time as morning samples would have been about 20 hr old at the time of testing and storage conditions might have modified the effect of production methods.

Rinses were taken at various times over the period of the survey. They were examined within a few hours of sampling.

Farm visits

Nineteen of the 20 farms whose milk had been regularly tested were visited and a record made of conditions on the farm, with special emphasis on factors which might be expected to contribute to the contamination of the milk by spore forming bacteria. When possible, rinses of the dairy equipment were taken.

Results

'Bittiness' and B. cereus *isolations*

Raw milk. The technique for estimating the *B. cereus* index enabled some check to be kept on the occurrence of bittiness in the milks under examination, as the 10 ml quantities were examined daily for signs of that condition. A precise end-point cannot easily be defined, as different degrees of bittiness can occur and in the later stages curd formation interferes; but the results obtained deserve comment.

The following assessments apply only to bittiness observed after 24 hr incubation at 22° and which was definite, in that it could not be dispersed by agitating the bottle containing the milk 25 times and persisted to the third day of incubation or was followed by complete coagulation.

Over a 12 month period, August 1955–July 1956, of 4,680 bottles examined, 1,498 (32%) showed definite bittiness on the first day. On the third day *B. cereus* was isolated from 2,076 (45%) of the total bottles (including some which did not show evidence of bittiness or clotting until the 2nd or 3rd day), but 257 (17·3%) of the *bitty* bottles did not show evidence of the presence of *B. cereus* when spotted on the 3rd day. It is possible that *B. cereus* had sometimes died out by this time but there was no reason to believe that this was common. Also *B. cereus* was seldom detected in samples showing late bittiness, i.e. not noticeable until the 3rd day of incubation.

The cause of this non-specific bittiness is not clear. Cultures isolated from such milks seldom caused any obvious change in the cream when inoculated into fresh pasteurized milk and none produced typical bittiness. Very occasionally other *Bacillus* species were isolated which were inhibitory for *B. cereus* var. *mycoides*, which might account for some cases of 1st day bittiness not yielding positive lecithinase reactions, but this was by no means common.

Pasteurized milk. Over a period of twelve months, 50 ml samples taken from bottled pasteurized milk from one dairy as delivered to the consumer, were incubated at 22°. After 24 hr they were examined for bittiness and a loopful of the cream was streaked on to egg yolk agar and the plates incubated at 22°. The results during different periods of the year are shown in Table 1. The incidence of *B. cereus* isolations was at its lowest during the spring and early summer months, and highest

during the winter months, when it was detected in almost all samples. When present it was not always the dominant organism; *Pseudomonas* and *Achromobacter* species giving a positive egg yolk reaction were frequently observed, either alone or together with *B. cereus*. Over the year, 95% of samples showed some degree of bittiness or a clot after 24 hr at 22°. In 72·5% the bittiness was marked and persisted when the milk was poured into hot tea, but in 16 instances (15%) *B. cereus* was not isolated, although there was marked bittiness. Two of these cases are of particular interest, as when the milk was poured into hot tea the bitty particles dispersed and large oily droplets appeared on the surface. In both instances a *Pseudomonas* species was isolated. This atypical bittiness was reproduced on inoculating a pure culture of the organism concerned into laboratory pasteurized milk free from *B. cereus*. With the remaining samples, no cause of bittiness could be identified.

Table 1. *The incidence of bittiness and* B. cereus *in bottled pasteurized milk from a single dairy*

Period	No. of samples	Percentages of samples	
		showing marked bittiness after 24 hr at 22°	from which *B. cereus* was isolated
March–June	30	57	40
July–October	44	84	79
November–February	34	73	96

B. cereus *indices of raw milks*

Farm milks. B. cereus was detected in 73% of 604 samples from 20 farms examined over a 12 month period, but only 8% of the samples showed a *B. cereus* index (i.e. presumptive *B. cereus*/100 ml) of more than 100 (Table 2). A *B. cereus* index of 1,800+ was occasionally recorded. *B. cereus* var. *mycoides* was detected in only 16% of the above samples. In 3·5% it was observed alone, but the index never exceeded 25.

Table 2. *The incidence of* B. cereus *in farm milks and the relationship of the colony count to the* B. cereus *index from 604 samples taken Aug.* 1955–*July* 1956

Range of colony count (/ml)	Percentage distribution of samples with *B. cereus* index ranges of:			
	<1	1–10	11–100	<100
$<10^3$	1·5	0·5	0·5	0
10^3–10^4	11	12	5·5	2
10^4–10^5	11	15	11·5	3
$>10^5$	3·5	11	9	3
Total	27	38·5	26·5	8

Bulk milks. Tanker milks received at London dairies from the West of England and Wales were sampled during the summer months June–September 1957. Of the

59 samples examined (Table 3), 10 showed an index of 1,800 +. A single bulk supply from the West delivered in cans to a South East Kent dairy was sampled in 1955. Of 6 samples taken in July, August and September, 5 showed indices of 1,800+. With 2 samples, dilutions beyond 1 in 10 were made and indices of 9,000 and 25,000 were obtained respectively.

Table 3. *The* B. cereus *indices of tanker milks sampled from June to September* 1957

Month	No. of samples	Mean *B. cereus* index
June	15	227
July	16	1,020
August	16	619
September	12	161

Seasonal variation. There was a marked seasonal variation in the *B. cereus* index. The mean monthly figures for all farm samples taken over a period of 2 years are shown in Fig. 1. Maximum numbers occurred in August and September when the mean index was well over 100. From September onwards there was a steady decline until April or May when the mean was less than 2, almost a hundredfold decrease.

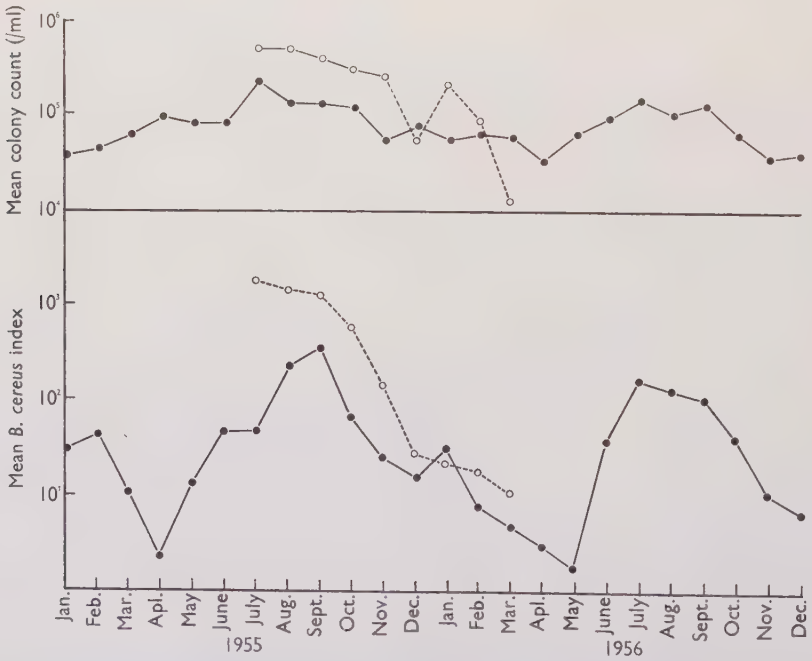

Fig. 1. Seasonal variation in the colony count and *B. cereus* index of raw milk supplies. Full circles, 20 farm supplies. Open circles, bulk churn supply.

From May to July or August the rise in numbers was rapid. The tanker milks showed a similar trend over the short period of sampling (Table 3). So also did the bulk supply in cans which was sampled from July 1955 to March 1956 (Fig. 1). The mean indices for these bulk milks exceeded those of the farm milks and were greater than 100 for a longer period. These figures are not however strictly comparable, as factors other than bulking may have played a part. In the case of the tanker milks, they were sampled in a different year from the others and while the farm milk supplies were from Northern and South-Eastern counties, all the bulk milk supplies were from the West country and Wales and neither would be representative of the country as a whole.

Individual farm results. The selected farms showed considerable variation in the *B. cereus* content of their milk. Samples from Farm no. 1 (Tables 4 and 5), taken over a 12 month period, gave no cases where the *B. cereus* index was over 10. In contrast, 67·5% of samples from Farm no. 20 showed an index of more than 10. A higher incidence of *B. cereus* in the summer months was noticeable with most supplies (Table 5). In the case of Farm no. 20, however, high indices were also obtained in the winter months.

Table 4. *A summary of the results of bacteriological analyses of milk samples taken from* 20 *farms between August* 1955 *and July* 1956

Farm no.	No. of samples	Percentages of samples giving			
		B. cereus index >10	Colony count/ml >10,000	Thermodurics/ml >1,000	Coli-aerogenes/ml >10
1	29	0	17	0	7
2	22	3	0	4	4·5
3	41	14·5	56	41·5	2·5
4	19	16	63	0	32
5	40	17·5	80	40	2·5
6	37	21·5	94·5	30	11
7	41	29	71	20·5	22
8	40	30	40	12·5	25
9	30	30	43·5	20	43·5
10	21	33·5	81	43	43
11	37	33·5	97	13·5	46
12	26	34·5	30	0	31
13	40	35	80	12·5	20
14	19	37	90	58	26·5
15	21	38	66·5	38	28·5
16	22	50	68	9	36
17*	39	56	97·5	90	74
18	31	58	29	3	42
19	31	66·5	64	6·5	35·5
20	40	67·5	100	75	55

* Milk 24 hr old when sampled.

Evening milk. For a 12 month period, samples of the previous evening's milk were taken at the same time as the morning samples in the case of three farms. The results given in Table 6 show that there was very little difference in *B. cereus* index between morning and evening samples at any of the three farms.

Table 5. *The mean monthly* B. cereus *indices of* 20 *farm milk supplies*

Farm no.	1955					1956						
	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	June	July
1	7	0	0	3	0	0	1	1	1	0	0	0
2	12	1	2	179	1	2	3	2	0	0	17	0
3	455	89	1	3	6	3	4	0	0	3	1	0
4	6	535	1	2	2	3	0	0	0	2	5	7
5	492	460	37	2	3	4	0	0	0	1	1	5
6	3	477	40	0	3	8	4	1	3	5	2	2
7	453	433	12	6	43	11	1	0	0	2	4	5
8	52	485	17	6	65	4	5	5	0	1	158	4
9	604	1,800+	9	14	35	18	13	3	2	3	3	3
10	10	901	835	1	5	12	4	2	4	0	6	350
11	11	20	9	16	16	8	7	3	5	5	5	4
12	117	0	40	22	17	8	15	7	2	0	11	3
13	94	455	36	94	13	15	2	2	0	8	0	13
14	808	906	62	5	3	3	1	5	0	2	45	35
15	910	450	62	175	65	6	3	0	2	1	1	70
16	1,600	365	126	8	6	18	5	5	11	7	840	1,800+
17*	12	512	52	33	19	23	22	31	0	3	6	677
18	150	9	125	31	6	19	7	15	3	1	6	29
19	22	462	150	25	12	30	9	15	14	0	6	88
20	110	30	36	52	50	388	50	9	6	0	14	739
Number of farms showing mean												
>10	17	16	14	10	10	9	4	3	2	0	6	9
>100	10	13	4	2	0	1	0	0	0	0	2	4

*Milk 24 hr old when sampled.

Table 6. *The* B. cereus *indices of evening and morning milk samples from* 3 *farms. February* 1956–*February* 1957

B. cereus index	No. of samples					
	Farm no. 1		Farm no. 15		Farm no. 16	
	p.m.	a.m.	p.m.	a.m.	p.m.	a.m.
<1	18	15	6	5	2	2
1–10	7	8	5	8	7	5
11–100	1	2	6	4	8	8
>100	0	1	2	2	4	6
Total samples	26	26	19	19	21	21

B. cereus *content of rinses*

Milk cans. Rinses were taken, using 500 ml of quarter-strength Ringer's solution, at various dairies in the north and south of the country and included hand and machine washed cans. The majority were examined using the dilution method, but in some cases the surface plate method was used.

The results for rinses tested by the dilution method are given in Table 7. Over the year, *B. cereus* was detected in 25% of cans but, as might be expected, there was considerable seasonal variation. During the three months July–September, *B. cereus* was detected in 44% of cans and a rinse count of over 5,000/can was obtained in 10·5% of cases. Using the surface plate method on rinses of 12 cans from a dairy where washing facilities were known to be poor, 3 gave a rinse count of more than 5×10^5 *B. cereus* cells/can.

Table 7. *The incidence of different ranges of count of* B. cereus, *and of high colony count at* 30°, *among milk can rinses*

Period	No. of rinses	Percentages of can rinses with				
		B. cereus count of:				Colony count
		<50	50–500	500–5,000	>5,000	$>5\times10^5$
Jan.–March	193	80	17	2·5	0·5	6
April–June	117	90	6	1	3	13
July–Sept.	134	56	21·5	12	10·5	33
Oct.–Dec.	54	74	16·5	7·5	2	26

Some evidence is available that milk cans may be an important source of *B. cereus*. In July 1957 routine samples from one of the farms (no. 1) started to show an erratic rise in both colony count and *B. cereus* index (Table 8). The dairy equipment had some milk stone on it and was descaled, and as the long milk tubes were beginning to perish they were replaced; rinses of the equipment did not suggest

Table 8. *The effect of contaminated milk cans on the* B. cereus *index and the colony count of the milk supply of Farm no.* 1

Date	Morning milk (3 hr old)		Evening milk (18 hr old)	
	Colony count at 30° (10^3/ml)	*B. cereus* index	Colony count at 30° (10^3/ml)	*B. cereus* index
5/7	488·0	0	>500·0	900
19/7	4·7	600	331·0	25
23/7	3·9	5	5·3	2
26/7	6·9	170	5·2	0
Long milk tubes renewed. Metal equipment descaled.				
29/7	383·0	3	5·5	13
13/8	18·7	50	23·2	130
19/8	2·4	110	3·6	1,800+
Cans sterilized with hypochlorite before use.				
26/8	0·5	5	1·5	5
6/9	3·0	0	17·3	0
10/9	3·1	2	11·7	11
16/9	2·0	0	2·8	13

Table 9. *Variation in the* B. cereus *count of can rinses taken at Farm no.* 1

B. cereus count/can		
Aug. 6	Aug. 7	Aug. 8
<50	<50	<50
100	<50	90,000+
45,000	<50	<50
850	100	250
100	100	350
<50	650	850

that the equipment was heavily contaminated with *B. cereus*, however. There was no obvious improvement in the samples as a result of these measures (Table 8), so the cans were examined. In general they were unsatisfactory and some showed a high *B. cereus* content (Table 9). The farmer was then advised to rinse all cans with hypochlorite (120 p/m) immediately before use, and following the adoption of this no further trouble was experienced in regard to either the colony counts or the *B. cereus* index (Table 8).

Milking equipment. Fifty-five rinses of all types of dairy equipment were taken during August and September at 17 of the farms and tested by the dilution method. In only one case, that of a cooler, was *B. cereus* detected in 10 ml of rinse. That this apparently low incidence of *B. cereus* was not due to efficient cleansing is shown by the colony counts/ml at 30°. The distribution was: under 5×10^3, 23%; 5×10^3–5×10^4, 22%; 5×10^4–5×10^5, 23%; over 5×10^5, 32%. Of 7 composite rinses (cluster, bucket, strainer, receiver and cooler) taken the following year at a single farm where methods were poor, 2 showed the presence of *B. cereus*, giving counts of 100 and 250/rinse respectively. 'First-milk-over' samples taken from the cooler at the same farm, following rinsing, showed indices ranging from 8 to 25, but these organisms could have been derived from the cows' udders, the air or other extraneous sources while the milk was being transferred from the bucket to the receiver, and need not necessarily have been derived from the equipment. Samples of foremilk indicated that *B. cereus* was frequently present in small numbers in or on the teats.

Relation of B. cereus *index to colony count at* 30°

There was no obvious relationship between the *B. cereus* index and the colony count at 30° of individual samples (Table 2). Samples with the lowest colony count normally had a low *B. cereus* index but the reverse did not hold for samples with high colony count. With milk can rinses the situation was similar.

With individual raw milk supplies (Table 4) the two best farms as shown by the *B. cereus* index also had low colony counts, and the worst farm was bad on both counts, but between these there was considerable variation.

The extent of the seasonal variation in the mean colony count for the 20 supplies differed noticeably in degree from that of the *B. cereus* index, though this was not so marked in the case of the single bulk churn supply (Fig. 1). There was, too, a difference in the time of year when numbers were rising or falling and also in the rate of this change, though the peak counts for both were obtained over approximately the same period. It appeared that colony count followed the average temperature curve more closely than did the *B. cereus* index. The same was true for can rinses; with these, minimum colony counts were obtained in the period January–March compared with April–June for the *B. cereus* index (Table 7).

No marked relationship was observed with the thermoduric and coli-aerogenes content of either individual milk samples or of supplies (Table 4).

The relation of the B. cereus *index to farm conditions*

A detailed comparison of conditions at the farm was not possible, but some of the factors which might be expected to have some influence on the bacteriological

quality of the milk are summarized in Table 10. As the condition of the cans might have affected the results, the dairies to which these supplies were delivered are indicated. Dairy AW was hand washing all cans at this time and constructional work at the dairy made it difficult for them to maintain a high standard. The condition of the cans supplied by the other dairies was fairly satisfactory. It will be seen from this Table that in general supplies from the better farms, judged on cleanliness, had a low *B. cereus* index, but the worst farms varied considerably; the same applied to udder washing. There was no evidence to show that the method of sterilizing equipment influenced the *B. cereus* index.

Table 10. *The relationship between farm conditions and* B. cereus *index*

The farms are listed according to the *B. cereus* index (see Table 4)

Farm no.	Colony count class††	Dairy code letters	Coli-aerogenes**	Clean-liness†††	Udder washing***	Method of cleansing
1†	I	SW	+	A	S	Steam
2	I	NH	+	A	S	Hypochlorite
3*	III	WA	+	C	Nil	Nil
4	III	NH	+++	B	FS	Hypochlorite
5	IV	WA	+	A	S	Hypochlorite
6	IV	WA	+	C	US	Hypochlorite
7	III	WA	++	B	Nil	Hypochlorite
8	II	NA	++	B	US	Steam
9	II	AW	+++	B	FS	Steam and Hypochlorite
10	IV	NH	+++	B	Nil	Hypochlorite
11	IV	WA	++++	Not visited		
12	II	AW	+++	A	S	Steam and Hypochlorite
13	IV	WA	++	C	US	Steam and Hypochlorite
14	IV	NH	++	B	FS	Hypochlorite
15	III	NH	++	B	US	Hypochlorite
16	III	NH	+++	C	?	Steam
17	IV	WA	++++	B	FS	Hypochlorite
18	II	AW	+++	B	?	Steam and Hypochlorite
19§	III	AW	+++	B	FS	Steam
20	IV	WA	++++	C	Nil	Hypochlorite

† Direct-to-can. * Hand milking 14 cows. Galvanized buckets with milk stone.
§ Auto-recorder. †† Percent samples $>10^4$/ml: 0–25%, I; 26–50%, II; 51–75%, III; >75%, IV.
**Percent samples showing presence in 0·1 ml: 1–15%, +; 16–30%, ++; 31–45%, +++; >45%, ++++.
††† A, good; B, average; C, poor. *** S, satisfactory; FS, fairly satisfactory; US, unsatisfactory.

Discussion

The counting methods devised and used in this investigation have yielded valuable information, but their limitations must not be overlooked.

Because *B. cereus* occurs in comparatively small numbers in milk a dilution method of counting had to be used, although it is known that such methods suffer from

inherent errors (Halvorson & Ziegler, 1933). In addition, heating had to be used as a selective process and this means that the index is almost certainly confined to spores alone. As incubation was limited to 3 days it is certain that some of the viable spores were not detected, as they had had insufficient time to germinate, and the index cannot be considered as a total spore count although it would include all of commercial importance. In the case of the surface plate method used for some rinses there was no heat treatment, so vegetative cells were not destroyed; but this lack of heating probably meant that germination of some spores was prevented or delayed. Therefore the final count obtained was probably not a total count of viable *B. cereus* vegetative cells and spores.

Another possible source of error is competitive inhibition during incubation, adversely affecting the *B. cereus* count, but the only direct evidence of this was that the spread of colonies of *B. cereus* var. *mycoides* on plates was prevented by certain other *Bacillus* spp.

The method used would not detect *B. cereus* strains which do not form lecithinase at 22°; but in an investigation concerned with bittiness, this is not important.

Finally, it must be remembered that the index or count depends on the assumption that any colony or spot which on egg yolk agar shows a typical surrounding opacity is in fact *B. cereus*. Fortunately, other organisms (e.g. *Pseudomonas* and *Achromobacter* spp.) known to give a positive reaction on the medium produce zones somewhat different in texture and can, with experience, readily be distinguished. In the genus *Bacillus*, so far as is known, the reaction is specific for *B. cereus* (McGaughey & Chu, 1948; Stone & Rowlands, 1952) and the assumption that the typical zone is in fact due to the growth of *B. cereus* is probably seldom incorrect.

Further investigation of the occurrence of bittiness in the absence of *B. cereus* is needed. The possibility of anaerobic bacteria such as *Clostridium welchii* playing a part cannot be ruled out. However, there is no doubt that *B. cereus* is the most important organism causing this fault.

Previous workers who have studied the incidence of mesophilic spore formers in raw milk supplies in connection with the sterilized milk industry (Ridgway, 1955; Franklin, Williams & Clegg, 1956), have used comparatively high temperatures of heating for the selection of sporing organisms. *B. cereus* spores will not normally survive temperatures above 90° for 30 min (Clegg *et al.* 1956) and so would not be included, but it is worth comparing their results with ours.

The seasonal variation observed by these workers differed from that observed in this investigation. There was a peak during winter and early spring for mesophilic spores which survived heating to 100°. With *B. cereus* numbers were at their lowest in spring and reached their peak in August and September.

In investigating individual farm supplies, Ridgway found no relationship between 'good' or 'bad' supplies (judged on methylene blue tests and colony counts) and those with good and bad keeping qualities following sterilization. This is in accordance with our findings that there was no marked relationship between the *B. cereus* index and colony count with individual supplies. The same also applied to thermoduric counts in both investigations.

The most important sources of spore formers in milk are still not certain. Our

results suggest that dairy utensils are not normally an important source of *B. cereus* but that milk cans may be, especially in the summer months.

Cans are obviously not the only source of *B. cereus*, as supplies delivered to the same dairy show such varying results. Ridgway (1955) suggested that the cow's udder (both the outer surface and the teat canals) was probably an important source of spore formers. We have not examined sufficient samples to say whether or not this is true for *B. cereus*, but udder infections with this organism have been recorded (Brown & Scherer, 1957). At Farm no. 1, where the *B. cereus* index of samples was normally consistently low, udder washing was good and teat cups were dipped in hypochlorite before the cluster was transferred from one cow to the next. If the outer surfaces of teats are an important source of *B. cereus*, this might account for the good results at this farm.

Other obvious sources of spore formers are hay, straw, dust, manure and mud. At two farms which showed a high *B. cereus* index in the winter, there is little doubt that hay and dust played a part. Hay was stored in or above the cowsheds, which were both exceptionally dirty and dusty. In summer, however, these factors will be less important, though mud might play an important part during a wet summer. The *B. cereus* content of soil taken from a grazing field at one farm, from April–August 1957, varied from 64×10^3–14×10^4/g wet weight; there was no significant seasonal variation. Samples of manure taken at the same time gave counts varying from 140 to 4,000/g.

The problem remains: how can the dairyman best limit the incidence of bittiness due to *B. cereus*? Egdell (1951) suggested that as much control as possible should be exercised over the raw milk supply, that plant management should be efficient, and that proper cold storage of the milk shou.d be ensured, not forgetting the consumer's larder. Stone & Rowlands (1952) and Stone (1952*a*) considered that infection of the milk was an insurmountable problem and that emphasis should be placed mainly on low storage temperatures before distribution, though in very hot weather they thought prevention of the fault might be virtually impossible. Our work has indicated how difficult it would be to exercise effective control over the raw milk supply by laboratory tests on farm milks at the times when bittiness is at its worst, i.e. during the summer months. During August and September, the majority of the 20 raw milk supplies tested showed a high *B. cereus* index and the results were liable to be erratic: in most cases they were not consistently high or consistently low over the peak period (Table 5).

Using a dilution method as described here as a routine would be impracticable under commercial conditions. A simplified method based on similar principles would be liable to large errors. It seems therefore that the dairyman's time would be better spent ensuring that he is not accentuating the trouble that already exists. It has been shown that cans may be a potent source of spores, especially in the summer months, and the remedy here is in the dairyman's hands. Bottle washers could easily be another source of spores; we have insufficient evidence of the frequency of this, but it is certain that many bottles returned by the consumer will contain large numbers of *B. cereus* spores; only really efficient washing will remove them.

Infection of milk with *B. cereus* spores appears to be inevitable. As they can

germinate and multiply rapidly at moderate temperatures, even in the presence of other spoilage organisms (Garvie & Stone, 1953), it is reasonable to assume that at least a ten-fold decrease in the initial numbers of *B. cereus* in raw milk supplies during the summer period would be necessary to bring about any marked improvement in the average rate of development of bittiness in pasteurized milk. There is no reason to believe that this can readily be achieved at present and efficient cold storage by processer, distributer and consumer remains the only really effective way of controlling the fault unless practical methods of stimulating spore germination in raw milk before pasteurization can be devised.

Grateful acknowledgment is made to all the staff of NAAS and NMTS laboratories who took part in this investigation, and we would also like to thank the dairies who so willingly allowed us sampling facilities.

References

Billing, E. & Luckhurst, E. R. L. (1957). A simplified method for the preparation of egg yolk media. *J. appl. Bact.* **20**, 90.

Brown, R. W. & Scherer, R. K. (1957). A report on two cases of acute mastitis caused by *Bacillus cereus*. *Cornell Vet.* **47**, 226.

Clegg, L. F. L., Sargeant, V., Franklin, J. G. & Auclair, J. E. (1956). The effect of lactenin on the growth of thermoduric bacteria in milk, with some observations on bitty cream. *Dairy Ind.* **21**, 128.

Egdell, J. W. (1951). Some bacteriological aspects of pasteurization and sterilization of milk. *J. Soc. Dairy Tech.* **5**, 29.

Franklin, J. G., Williams, D. J. & Clegg, L. F. L. (1956). A survey of the number and types of aerobic mesophilic spores in milk before and after commercial sterilization. *J. appl. Bact.* **19**, 46.

Halvorson, H. O. & Ziegler, N. R. (1933). Application of statistics to problems in bacteriology. III. A consideration of the accuracy of dilution data obtained by using several dilutions. *J. Bact.* **26**, 559.

Garvie, E. I. & Stone, M. J. (1953). The associative growth of *Bacillus cereus* and *Streptococcus lactis* in milk. *J. Dairy Res.* **20**, 29.

McGaughey, C. A. & Chu, H. P. (1948). The egg yolk reaction of aerobic sporing bacteria. *J. gen. Microbiol.* **2**, 334.

Ridgway, J. D. (1955). Some recent observations on the bacteriology of sterilized milk. *J. appl. Bact.* **18**, 374.

Stone, M. J. (1952*a*). The effect of temperature on the development of broken cream. *J. Dairy Res.* **19**, 302.

Stone, M. J. (1952*b*). The action of the lecithinase of *Bacillus cereus* on the globule membrane of milk fat. *J. Dairy Res.* **19**, 311.

Stone, M. J. & Rowlands, A. (1952). 'Broken' or 'bitty' cream in raw and pasteurized milk. *J. Dairy Res.* **19**, 51.

Explanation of Plate

Determination of the *Bacillus cereus* index of raw milk.

(a) Inoculated bottles and tubes after 3 days at 22°. The letters a, f and k identify the bottle and tubes which were 'spotted' on the confirmatory medium (see below, b).

(b) Egg yolk agar plate inoculated from the above bottles and tubes after 24 hr at 22°.

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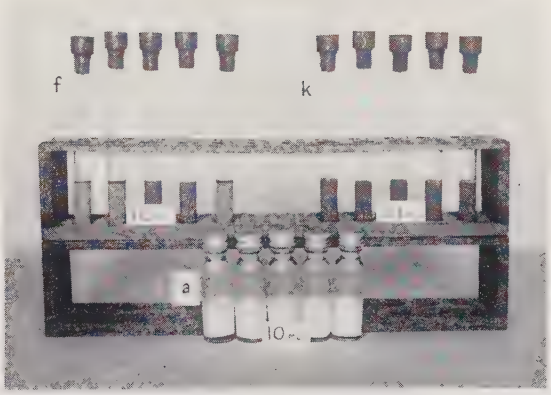
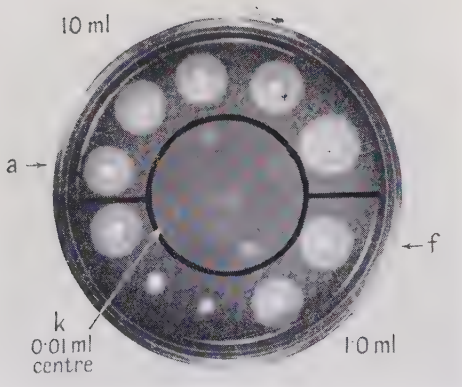


(*a*)



(*b*)

EVE BILLING AND W. A. CUTHBERT — 'BITTY' CREAM: THE OCCURRENCE AND SIGNIFICANCE OF *BACILLUS CEREUS* SPORES IN RAW MILK SUPPLIES. PLATE 1

Medrek, T. F. & Barnes, E. M. (1958). *J. appl. Bact.* **21** (1), 79.

A NOTE ON THE GROWTH OF *STREPTOCOCCUS BOVIS* IN THALLOUS ACETATE AND TETRAZOLIUM MEDIA

By T. F. MEDREK and ELLA M. BARNES

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It was reported by Barnes (1956*a*,*b*) that the reduction of tetrazolium at pH 6·0 could be used to separate *Streptococcus faecalis* and its variants *zymogenes* and *liquefaciens* from other faecal streptococci of Lancefield group D, such as *Streptococcus faecium*, *Strep. durans* and *Strep. bovis*, which did not reduce tetrazolium.

Studies on a number of reference and freshly isolated strains of *Strep. bovis* now indicate that the *Strep. bovis* strain originally used in the tests was only representative of a proportion of the strains comprising this species. Lab-Lemco peptone glucose broth (pH 6·0) containing 0·1% of thallous acetate can be used for the primary isolation of all the *Strep. bovis* strains, but only some of these grow well on thallous acetate tetrazolium agar, producing white or pale pink colonies. The other strains grow feebly on this medium, producing minute red colonies. *Strep. bovis* strains in broth at pH 6·0 reduce 1–15% of the tetrazolium to formazan. This differs from most strains of *Strep. faecium* and *Strep. durans*, which do not reduce tetrazolium at this pH. Reduction by *Strep. faecalis* and its variants *liquefaciens* and *zymogenes* is generally greater than 50%. The difference in the behaviour of *Strep. bovis* strains on tetrazolium agar is also reflected in a number of other properties of the organisms concerned, which are now being investigated.

This work was done while the senior author held a Fulbright Scholarship.

References

Barnes, E. M. (1956*a*). Tetrazolium reduction as a means of differentiating *Streptococcus faecalis* from *Streptococcus faecium*. *J. gen. Microbiol.* **14**, 57.

Barnes, E. M. (1956*b*). Methods for the isolation of faecal streptococci (Lancefield group D) from bacon factories. *J. appl. Bact.* **19**, 193.

(*Received* 13 *February*, 1958)

Elsworth, R., Capell, G. H. & Telling, R. C. (1958.) *J. appl. Bact.* **21** (1), 80–85.

IMPROVEMENTS IN THE DESIGN OF A LABORATORY CULTURE VESSEL

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SUMMARY: The application of a radial mechanical shaft seal, and other improvements to a 2–3 l. capacity culture vessel, are described.

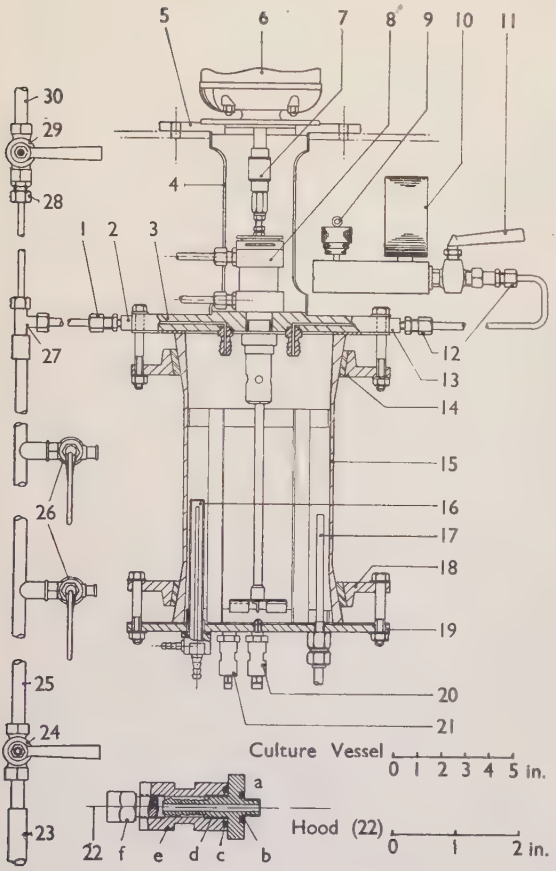
To a worker confronted with the need to use deep culture apparatus it is not unusual for the early microbiological studies to be overshadowed by problems of culture vessel construction and operation. An earlier paper (Elsworth, Meakin, Pirt & Capell, 1956) described a culture vessel, examples of which have operated continuously for periods exceeding 1,000 hr. Among its imperfections is the stuffing box type of shaft seal, which is not always tight when the vessel is operated under pressure. This imposes the need to dismantle the vessel in order to sterilize it in an autoclave. This paper describes the use of a mechanical seal, now generally regarded as superior to the stuffing box type on account of better sealing capacity, improved reliability and need of less maintenance. The vessel may now be sterilized under pressure with internal steam, which reduces work in preparing the vessel for use. Sterilization at atmospheric pressure with steam containing formalin vapour is described. This method can be applied with advantage to vessels fitted with the stuffing box seal described in the earlier paper.

An improved assembly

Each time the vessel described in the earlier paper is sterilized the drive has to be dismantled. On re-assembly, realignment of the drive is based on visual judgment and if done incorrectly the motor may be overloaded or the journal bearings in the shaft seal unduly stressed. In the modified design the motor and seal bodies are accurately located in machined spigot fits, and misalignment of the shafts is impossible. Once assembled, the motor and stirrer shafts need not be parted. To clean the vessel or change an impeller, only the glass body need be removed by breaking one joint. To sterilize the vessel no dismantling whatever is required.

The alternative construction is shown in Fig. 1. The distance piece (4), fabricated by welding, is supported from its upper flange (5) on a stand, of which only the supporting surface is shown. The motor (6) or the driving shaft housing fits concentrically into the upper flange (5) of the distance piece (4). Similarly the stirrer seal (8) fits concentrically into the lower flange (3) of the distance piece. In this way the shafts are in alignment. They are connected by the flexible coupling (7). The body of the culture vessel is attached to the lower flange (3) of the distance piece by a

Fig. 1. General arrangement of culture vessel (diagrammatic).



1, connection for exit air, unequal male stud coupling (brass), $\frac{1}{4}$ in. o.d. tube, $\frac{1}{8}$ in. B.S.P. male thread, part no. D43/16/081 (Simplifix Couplings Ltd., Maidenhead, Berks); 2, reducing bush, $\frac{1}{8}$ in. B.S.P. female, $\frac{1}{4}$ in. B.S.F. male; the joint at male end is made with a toroidal 'O' ring (Specification, 1951, Table 5, seal no. O.S.7); 3, lower flange of distance piece which forms lid of culture vessel; the holes through the edge of the flange are drilled $\frac{1}{8}$ in., the connections at each end are $\frac{1}{4}$ in. B.S.F. female thread; 4, tubular distance piece, 4 in. o.d. $\times$ 14 S.W.G.; 5, upper flange of distance piece; 6, flange mounted motor (Elsworth *et al.* 1956); 7, 'Resilla' flexible coupling no. 1 (J. H. Fenner & Co. Ltd., Hull); 8, body of stirrer seal showing slot for discharge of gland leaks; 9, relief valve set to blow at 15 lb/in.^{2}; 10, pressure gauge; 11, AB10 sleeve packed brass cock, pattern no. 500,011 (R. Klinger Ltd., Sidcup, Kent); 12, see 1; 13, see 2; 14, backing flange, 6 in. (Specification, 1955, Table 5); 15, 12 in. length of 6 in. '*d*' pipe section (Specification, 1955, Table 2) jointed with $\frac{1}{16}$ in. thick C.A.F. gaskets, ref. no. CG/6, protected with Fluon sheaths, ref. no. FS/6 (Q.V.F. Ltd., Fenton, Stoke-on-Trent, Staffs.); 16, cooling finger, sheath dimensions based on Fig. 2(c) of Elsworth *et al.* (1956); the $\frac{3}{16}$ in. o.d. hypodermic internal tube is an interference fit into the exit connection which is screwed $\frac{1}{4}$ in. B.S.F. and sealed with an 'O' ring (Specification, 1951, Table 5, seal no. O.S.7), and flats for a $\frac{9}{16}$ in. B.S. spanner are provided on the base; 17, sheathed resistance thermometer element (Foster Instrument Co. Ltd., Letchworth, Herts, described in the firm's publication Book 134, Electrical Resistance Thermometers, p. 32), threaded $\frac{1}{4}$ in. B.S.P. male and sealed to the base of the vessel with a 'Seloc' washer, ref. no. GD.1321/8 (Dowty Seals Ltd., Ashchurch, Glos.); 18, see 14; 19, base of culture vessel; 20, air inlet; 21, sample point; 22, details of hooded branch connection, (a) $\frac{1}{4}$ in. B.S.F. thread, (b) Specification (1951), Table 5, seal no. O.S. 7, (c) Specification (1951), Table 5, seal no. O.S. 9, (d) $\frac{1}{8}$ in. B.S.P. thread, (e) brass body, $\frac{3}{4}$ in. o.d. $\times 1\frac{3}{8}$ in. long, with flats on exterior for $\frac{1}{4}$ in. B.S. spanner, (f) radiator vent valve, $\frac{1}{8}$ in. B.S.P. male; 23, hose connection to atmosphere; 24, AB12, $\frac{1}{4}$ in. bore forged steel cock, screwed $\frac{1}{4}$ in. B.S.P. female (R. Klinger Ltd., Sidcup, Kent); 25, manifold, $\frac{1}{4}$ in. class B pipe (Specification, 1947*b*) made of stainless steeel (Specification, 1947*a*, En. 58B), with welded sockets as branch connections; 26, AB10 sleeve packed brass cocks, pattern no. 500,013 (R. Klinger Ltd., Sidcup, Kent); 27, tee-piece made up of $\frac{1}{4}$ in. B.S. pipe socket and Simplifix male stud tee, $\frac{1}{4}$ in. o.d. tube, $\frac{1}{4}$ in. B.S.P. male thread, part no. D51/16/161/16; 28, Simplifix equal male stud coupling, $\frac{1}{4}$ in. o.d. tube, $\frac{1}{4}$ in. B.S.P. male thread, part no. D43/16/161; 29, see 24; 30, steam supply fitted with safety valve to blow at 20 lb/in.^{2}

The exit air line is made of copper and stainless steel. All parts in contact with culture are made of Ni-Cr stainless steel (Specification, 1947*a*, En. 58B), except the stirrer shaft which is made of plain Cr steel (Specification, 1947*a*, En 56A or En. 56AM).

standard industrial glass pipeline backing flange (14). Improved accessibility results from inserting branch connections (1, 12) through the edge of the lower flange (3) which forms the lid of the culture vessel. The base (19) of the vessel is attached by another pipeline flange (18). The complicated cooling jacket in the original version is replaced by a cooling finger (16) which is easier to make and fit, and has a larger cooling area than the original. A smaller, quicker response, sheathed resistance thermometer element (17) is now used. This is inserted directly into the base (19) and dispenses with a pocket. The air supply pipe (20) is now a standard small branch connection. An exit air pipe is connected at the branch connection (1), and a relief valve assembly (9, 10, 11), used during sterilization, is connected at (12).

A mechanical seal for the stirrer shaft

As a rotary shaft seal the packed stuffing box is obsolete and is being replaced in most applications by the so-called radial mechanical seal. Wood (1954) has summarized the limitations of the former, and stated the basis for the successful design and operation of mechanical seals. Hedén, Malmgren, Sundström & Törnqvist (1952) have already proposed their use in pilot scale culture vessels and they are widely used on large scale plant.

On the small scale the particular undesirable features of a packed stuffing box seal are: first, that in reducing leakage by tightening up the gland there is always the chance of overloading the fractional horse-power driving motor; second, to get satisfactory agitation on this scale, the high shaft speeds used cause rapid wear of the shaft in the stuffing box, and result in reduced shaft life; third, the performance of stuffing box seals, particularly on small diameter shafts, is erratic and there is frequent need for adjustment.

Radial mechanical seals overcome all these disadvantages. In particular, power absorption is constant, shaft wear is eliminated and above all there is no need for adjustment. The seal is made between replaceable counter-faces, one of which is sealed to the body of the vessel and is thus stationary. The other is sealed to the shaft and thus rotates. The two form a self-lubricating pair. In this example the stationary face is metal, and the rotating face a plastic bearing material. The faces are lapped flat with great accuracy, and during use are immersed in a liquid which cools them. When wear on the counter-faces eventually results in leakage, the seal is repaired by fitting new or reconditioned counter-faces. New components cost only about £1. There are several types of mechanical seal and the one selected for this application is a bellows type (Crane Packing Ltd., Slough). It is described as the 'Type 6 compact seal for $\frac{3}{8}$ in. diam. shaft, material specification BT.2.4.1'. For further details the company's booklet 'Mechanical Seals' should be consulted. The code reference defines materials of construction which withstand both occasional sterilizing with steam and prolonged immersion in condensed steam, which is used to cool them.

The seal shown in Fig. 2 is of the double type effective against secondary and laboratory infection. The lower seal is made between counter-faces (14, 16) and prevents laboratory infection (i.e. egress of culture). The upper seal is made between counter-faces (8, 10) and prevents secondary infection (i.e. ingress of atmospheric

contamination). The body (7) is sealed to the vessel lid by the 'O' ring (17). The body contains ports (15, 9) for entry and exit of the cooling water. At the lower end a stationary counter-face (16) is inserted and sealed with a gasket. At the top the bearing housing (3) carries the second stationary counter-face (8). The bearing housing (3) screws into the seal body and makes a seal at an 'O' ring (6). Below the bearing are two slots (5) through which in the event of a leak the cooling water discharges. If the slots are omitted, leaking water discharges through the ball race. The rotating counter-faces (10, 14) are attached and sealed to the respective rubber bellows (11, 13), which in turn are sealed and attached to the shaft. The power consumed at the counter-faces is determined by the compression of the bellows, which contain a spring (not shown). A muff (12) is fixed to the shaft and separates the bellows. Its position ensures that the strain on each bellows is the same. The strain is determined by dimension 'd'. The body (7) is secured to the vessel lid by the shaft bush support (18).

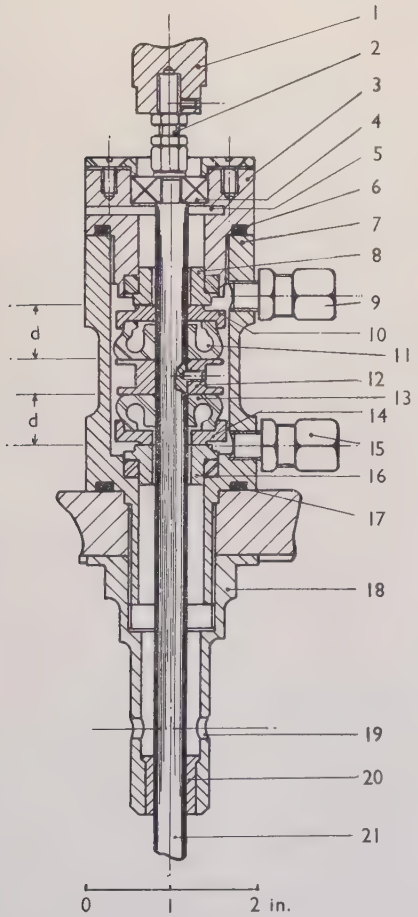


Fig. 2. Radial mechanical seal.

1, flexible coupling (see Fig. 1, 7); 2, stirrer shaft, screwed $\frac{1}{4}$ in. B.S.F.; 3, bearing housing (brass), threaded 1·334 in. major diameter × 24 t.p.i. Whitworth form, British Standard close fit (Handbook, 1946); 4, ball journal BRL 007, U.K. fit on mating parts (Handbook, 1946); 5, two slots for gland leaks (only one shown); 6, 'O' ring seal (Specification, 1951, Table 5, seal no. O.S. 27); 7, seal body; 8, stationary counter-face sealed to body with a square-section gasket; 9, exit port for condensate, fitted with unequal stud coupling, $\frac{1}{8}$ in. B.S.P. male thread, $\frac{1}{4}$ in. o.d. copper tube, part no. D43/16/081 (Simplifix Couplings Ltd., Maidenhead, Berks.); 10, rotating counter-face; 11, bellows; 12, shaft muff; 13, as 11; 14, as 10; 15, entry port for condensate, as 9; 16, as 8; 17, as 6; 18, shaft bush support, threaded 1 in. major diameter × 20 t.p.i. Whitworth form, B.S. close fit; 19, leak holes; 20, Ferobestos bush, grade LF37 (see Elsworth *et al.* 1956, for further details); 21, $\frac{3}{8}$ in. diameter ground shaft. A bellows working length of d = 0·613 in. is satisfactory.

The shaft and Ferobestos bush need renewing after about 4,000 hr operation. This is due to a weakness in the design, probably remediable by using other material either for the complete shaft or at its bearing surface. Correctly used. the seal components last for years. Examples in use are still serviceable after more than two years' operation.

Although mechanical seals are described as 'tight', if unsterile cooling water is used the risk must be accepted of some leakage into the vessel, which will result in secondary infection. The use of sterile cooling water at greater than atmospheric pressure and at a positive pressure (minimum, 1 lb/in.^{2}) relative to the contents of the vessel, will ensure that leaks across the counter-faces, which cannot always be detected visually, will consist of sterile material. It is convenient in our case to use condensed steam as cooling fluid. This is drawn from a steam supply whose pressure varies from 20–30 lb/in.^{2} The strained steam is condensed and cooled by passing through a 3 ft length of $\frac{1}{2}$ in. B.S. class B pipe (Specification, 1947*b*) made of stainless steel (Specification, 1947*a*, En 58B) fitted with a water jacket. This produces 100 ml/min of cool condensate which is fed to one seal. The amount used could probably be reduced. If a steam supply is not available, sterile water from a reservoir above the sealing chamber will do. For a scheme of condensate preparation see Gibbons & Ulrich (1956). To get clean condensate, a strainer in the steam supply and the use of non-ferrous or stainless steel fittings are essential.

Sterilization

Condensate supply and sealing chamber. The cooling water is shut off and drained from the jacket of the condensate maker. A cock on the exit port of the sealing chamber is opened wide. By regulating the steam at a cock between the condensate maker and the entry to the sealing chamber, the former is treated with steam under mains pressure and the sealing chamber treated with steam at atmospheric pressure for 30 min. The seal components are steamed at atmospheric pressure in order to reduce the temperature to which the bellows are subjected. Once sterilized, condensate under pressure, regulated by the cock on the exit port, is passed through the sealing chamber at all times, even when the shaft is not running. In this way the sealing chamber is substantially at the pressure of the steam main. It is not necessary to re-sterilize the sealing chamber each time the vessel is sterilized.

Pressure sterilization of the culture vessel. Fitted with the improved shaft seal, the vessel is suitable for operation at a maximum working pressure of 25 lb./in.^{2} (see Specification, 1955) and may therefore be sterilized with internal steam. The branch connection on the lid of the culture vessel used for the exit air line (Fig. 1, 1) has a permanent steam connection and is fitted with cocks as shown. Another branch (12) is connected by $\frac{1}{4}$ in. o.d. copper pipe to a manifold containing a relief valve (9) of the type fitted to a domestic pressure cooker, and a pressure gauge (10). The remaining branch connections are fitted with hoods (22) which terminate in vent valves of the type used on central heating radiators. The air inlet (20) and the sample point (21) in the base of the vessel are fitted with similar hoods.

During sterilization the vessel is surrounded by a hardboard screen. Before steam is turned on, a check is made that there is condensate pressure in the sealing chamber of the shaft. To sterilize, the vessel is treated in the normal way with internal steam at 15 lb/in.^{2} for 20 min, though it may be noted that Finn (1956) quoted 7 min at this pressure as the minimum time for 99·99% destruction of spores. When the steam supply is turned off, all vents except cock (11) are shut. Just as the internal pressure reaches zero, cock (11) on the relief valve manifold is closed

and the hood on the air inlet (20) is removed. A sterile bacterial filter (Elsworth *et al.* 1956) attached to a length of sterile rubber tube is then connected aseptically to the air inlet and a stream of air passed through the vessel and purged at cock (24). When required for use, sampling apparatus as described in the earlier paper is connected to branch (21). Sterile medium is then added through a hooded branch connection on the lid (not shown). Any remaining connections on the lid are kept closed or used as required.

Treatment with formalin-steam. The two methods described below are alternative to sterilization with steam under pressure. By using either of them, the vessel described in the earlier paper may be sterilized without dismantling. The first method is satisfactory for vegetative cultures. It is carried out by first rinsing the vessel with water and then adding 5 ml of formalin solution (37–41% w/v). Steam at atmospheric pressure is then passed into the vessel and purged at all vents for 2–3 hr. Sterile air is then passed through the vessel until it is required. The second method is suitable for sterilizing spore cultures (e.g. *Bacillus subtilis*). Formalin solution is evaporated in a steam heated flash boiler and the vapour is mixed with steam to give a formalin concentration of 1 mg/g measured on a sample of condensate. This formalin-steam mixture, at atmospheric pressure, is then passed through the vessel for 20 min, again purging at all vents. The formalin supply is then shut off and pure steam is passed for 10 min to remove residual formalin. As before, sterile air is passed through the vessel until it is required.

The development of this culture vessel owes much to ideas and work by Messrs. Stockwell, Ranger and Duckett. We thank Mr. R. S. Sweett, of Crane Packing Ltd., for criticism of the description of the seal.

References

Elsworth, R., Meakin, L. R. P., Pirt, S. J. & Capell, G. H. (1956). A two litre scale continuous culture apparatus for micro-organisms. *J. appl. Bact.* **19**, 264.

Finn, R. K. (1956). Principles of industrial sterilization. In a Symposium on '*Biochemical Engineering*' sponsored by the American Institute of Chemical Engineers (Terre Haute, Indiana).

Gibbons, E. J. & Ulrich, J. (1956). Condensate provides pump seal liquid. *Chem. Engng* **63** (1), 216.

Handbook (1946). Handbook No. 2, *British Standards for Workshop Practice*. London: British Standards Institution.

Hedén, C-G., Malmgren, B., Sundström, K. E. & Törnqvist, B. (1952). Studies on the cultivation of micro-organisms on a semi-industrial scale. *Acta path. microbiol. scand.* **30**, 284.

Specification (1947*a*). *British Standard Specification for Wrought Steels.* No. 970. London: British Standards Institution.

Specification (1947*b*). *British Standard Specification for Steel Tubes and Tubulars.* No. 1387. London: British Standards Institution.

Specification (1951). *British Standard Specification for Dimensions of Toroidal Sealing Rings.* No. 1806. London: British Standards Institution.

Specification (1955). *British Standard Specification for Glass Pipeline and Fittings.* No. 2598. London: British Standards Institution.

Wood, T. H. (1954). Mechanical shaft seals in the chemical industry. *Trans. Instn chem. Engrs, Lond.*, **32**, 73.

(*Received* 14 *February*, 1958)

Harris, N. D. & Richards, J. P. (1958). *J. appl. Bact.* **21** (1), 86–93.

THE FAILURE OF PHENOL TREATED *ESCHERICHIA COLI* TO GROW ON MEMBRANE FILTERS

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SUMMARY: Counts of *Escherichia coli* were done on nutrient agar (control), on membrane filters on nutrient agar and on membrane filters on filter paper pads. With untreated bacteria counts were similar under all conditions, though membrane filters on nutrient agar tended to give slightly low counts. Phenol treated bacteria gave much lower counts when membrane filters were used: the mean counts for 3 strains of the test organism with filters on nutrient agar varied from 35–65% of the control, while counts with filters on filter paper pads were somewhat lower, varying from 30–47% of the control. The low counts on membrane filters on filter paper pads were not due to adsorption of phenol by the filters or to a low concentration of nutrients in the growth medium.

Membrane filters are being used increasingly for counting bacteria, particularly in the examination of water, and the counts on these filters have been compared with those obtained by conventional methods. Kabler (1954) and Hope & Neill (1956) compared membrane filter and 'most probable number' counts on waters and found that the results were generally in agreement. Goetz & Tsuneishi (1951), using 4 different species, and Taylor, Burman & Oliver (1955), counting water samples, also found that counts using membrane filters agreed with those on agar plates. Similar agreement between these two methods was reported by Slanetz, Bent & Bartley (1955) using *Streptococcus faecalis*. However, Shipe & Fields (1954) reported membrane counts of *Escherichia coli* which were consistently lower than agar plate counts, both with untreated suspensions and with cells exposed to copper and zinc sulphates. Similarly Shipe & Cameron (1954) obtained lower counts with membrane filters than with the 'most probable number' method from waters which contained toxic substances. Membrane filter counts which were slightly lower than those on agar plates were reported by Portner, Mayo & Kaye (1954) with untreated bacteria, but with bacteria exposed to the action of formaldehyde these workers found higher counts on the membrane filters.

In view of these somewhat contradictory reports it was thought that it would be useful to see whether membrane filters had any advantages over nutrient agar for the cultivation of *E. coli* cells which had been exposed to phenol.

Materials and Methods

Media. The nutrient broth used routinely was prepared from 'Oxoid' Lab-Lemco granules (CM15) at a concentration of 16 g/l. When necessary, media were solidified with 15 g/l. of powdered New Zealand agar (Davis Gelatine Ltd.).

Counting techniques. Viable counts were done by the general technique of the surface viable method (Miles & Misra, 1938) using the dropping pipette described by Cook & Yousef (1953). The usual practice of filtering the suspension through the membrane was not done, since direct comparison with the nutrient agar counts was desired. Ten drops were counted from each suspension.

The membrane filters (8 cm) were sterilized in accordance with the suppliers' recommendations and dried overnight at 40°. They were incubated on a Whatman no. 17 filter paper pad sterilized and dried similarly to the membranes. For use the pad was transferred to a 9 cm Petri dish and 5 ml of nutrient broth added to it. A dry membrane filter was then placed on the pad, care being taken to obtain uniform contact, and a few minutes were allowed for wetting of the membrane. The volume of broth was found to be critical: with smaller volumes the drop failed to spread to the same extent as on nutrient agar and low counts were obtained due to overcrowding, while larger volumes also gave low counts due to confluent growth. Similar observations have been made by Powers, Ehret & Bannon (1957). For incubation the bottom of the dish, containing the membrane on the pad, was inverted over moistened filter paper in the lid to prevent dehydration. This procedure was recommended by McKee *et al.* (1953); however, Slanetz, Bent & Bartley (1955) found that humidity had little influence on coli-aerogenes counts on membrane filters. These optimum conditions were established using suspensions of untreated bacteria: experience showed that they needed to be established afresh for different batches of membrane filters or different media.

Organisms. Three strains, A, B and C, of *E. coli* (all type I, 44°+, IMViC, ++−−) were used. Strain A, isolated from water in 1951, was used routinely. Strain B had been isolated very recently from water, while Strain C was an old laboratory strain.

The biochemical reactions of all three strains were similar except that they differed in their ability to ferment sucrose and raffinose and in their action on litmus milk.

For use the growth from a 24 hr culture on nutrient agar was suspended in distilled water, standardized nephelometrically and 2 ml added to 8 ml of a 1·25% (w/v) phenol solution so that the final concentration of phenol was 1% (w/v). After a suitable time of contact this reaction mixture was diluted $1:10^4$ with distilled water, one drop usually giving a countable number of colonies. Untreated suspensions were diluted to give similar counts.

Counts were done on (a) 'overdried' nutrient agar, (b) membrane filter on 'overdried' nutrient agar and (c) membrane filter on filter paper pad.

Results

The results given in Table 1 for untreated suspensions show that the mean viable counts on the membranes were not very different from those on the nutrient agar control. Counts on membrane filters on nutrient agar were invariably slightly lower than the control counts, the mean being 90% of the count on nutrient agar.

Phenol treated suspensions behaved very differently: membranes on agar gave lower counts than nutrient agar alone in 4 out of 5 trials, and membranes on filter

pads invariably gave still lower counts, the mean responses being 62% and 22% of the control respectively (Table 1). Only in Trial 12 did the count of phenol treated organisms on the membrane on nutrient agar exceed that on agar alone, and then the difference was small.

Table 1. *Viable counts of untreated and treated* E. coli *strain A on membrane filters and nutrient agar*

	Mean viable count/drop					
	Untreated			Phenol treated		
Trial no.	Nutrient agar (control)	Membrane on nutrient agar	Membrane on filter pad	Trial no.	Nutrient agar (control)	Membrane on nutrient agar	Membrane on filter pad
1	38·0	—	34·9	1	68·3	—	4·0
2	30·8	—	38·7	2	57·4	—	19·0
3	35·4	—	40·0	3	58·8	—	22·4
4	41·8	—	37·0	4	4·1	—	0·2
5	18·4	16·5	—	5	2·8	—	1·7
6	24·5	19·4	—	6	36·5	—	2·9
7	27·0	22·2	—	7	146·0	—	29·9
8	23·1	20·9	—	8	33·6	—	1·0
9	13·4	13·2	—	9	16·3	9·9	—
10	21·7	20·2	—	10	2·6	1·5	—
11	16·3	15·7	—	11	22·7	6·0	—
12	33·8	30·2	—	12	81·7	83·2	—
				13	85·7	52·2	—
Mean response (% of control)	100	90	105	Mean response (% of control)	100	62	22

To confirm these results the experiments were repeated, this time all three incubation conditions being compared simultaneously. Further trials were also done with strains B and C to ensure that the effects were not peculiar to the original test strain. The general trend of the previous observations was fully confirmed (Table 2).

Whatever the incubation conditions the counts of untreated cells of all three strains were very similar, but once again the mean counts with membrane filters on nutrient agar were slightly lower than on the control. When all the results for strain A from Tables 1 and 2 were combined the mean count on the membrane on nutrient agar was 92% of the control and this difference is significant ($t=3{\cdot}282$, $P<0{\cdot}01$). No significant differences in count of untreated cells from the control could be demonstrated with the other strains, or when membrane filters were incubated on filter pads.

In connection with the results for strain A on membranes on nutrient agar, it is interesting that Clark & Kabler (1952) stated that membranes should not be incubated on media containing agar, though they gave no evidence to support their statement. Also Gohar & Eissa (1957) found that, except with waters of high purity, membrane filters incubated on agar gave lower counts than direct plating or presumptive coli-aerogenes tests. However, Slanetz & Bartley (1957) found higher counts and larger colonies of enterococci when membrane filters were incubated on

an agar medium than when filter pads were used, and Buttiaux, Muchemble & Leurs (1953) used solid media routinely with membrane filters.

Table 2. *Viable counts of three strains of untreated and treated* E. coli *on membrane filters and nutrient agar*

Strain	Mean viable count/drop							
	Untreated				Phenol treated			
	Trial no.	Nutrient agar (control)	Membrane on nutrient agar	Membrane on filter pad	Trial no.	Nutrient agar (control)	Membrane on nutrient agar	Membrane on filter pad
A	1	18·5	20·0	20·4	1	89·6	62·4	38·0
	2	18·6	20·0	19·5	2	131·4	80·6	46·3
	3	23·1	21·6	20·9	3	72·1	59·1	44·8
	4	41·1	34·7	39·5	4	62·1	37·9	19·8
	5	33·8	28·7	32·2	5	11·3	3·8	1·8
	—	—	—	—	6	41·1	22·0	14·4
	—	—	—	—	7	79·9	77·3	71·5
Mean response (% of control)		100	96	99		100	65	45
B	1	27·1	27·2	32·5	1	127·7	75·5	37·6
	2	22·0	19·2	19·3	2	87·3	57·2	65·8
	3	33·0	35·0	33·6	3	44·6	22·0	15·9
Mean response (% of control)		100	98	103		100	58	47
C	1	31·1	33·7	33·9	1	32·1	4·9	10·3
	2	29·3	24·7	32·0	2	53·4	19·5	6·9
	3	33·7	30·2	29·8	3	56·2	29·0	24·5
Mean response (% of control)		100	94	102		100	35	30

Table 3. *The* 't'*-test values obtained from the data in Tables* 1 *and* 2 *for treated organisms*

Strain	Method of incubation of membrane filter	Mean difference from control count		*t*	*P*
		No.	%		
A (Table 2 data only)	Nutrient agar	−20·6	−35	3·42	<0·02
	Filter pad	−35·8	−55	3·53	<0·02
A (Data from Tables 1 & 2)	Nutrient agar	−16·7	−36	3·77	<0·01
	Filter pad	−38·6	−67	4·87	<0·001
B	Nutrient agar	−35·0	−42	3·93	*c.* 0·06
	Filter pad	−46·8	−53	2·14	*c.* 0·17
C	Nutrient agar	−29·4	−65	13·11	<0·01
	Filter pad	−33·3	−70	4·64	*c.* 0·04

Counts of treated cells were invariably lower on membrane filters than on nutrient agar, no matter whether the filters were incubated on nutrient agar or on filter pads, the latter method giving the lower counts in all trials except one. Application of the '*t*'-test showed that the differences with strains A and C were significant (Table 3). When all the results for strain A from Tables 1 and 2 were combined this comparison

was even more striking. There was some variation in response with strain B and the '*t*'-test did not show that the observed counts were significantly less than the controls. Despite this absence of formal statistical proof the fact is that lower counts were observed in all trials with membrane filters (Table 2), and the similarity of the response to that of the other strains is very striking.

The effect of concentration of the medium

Several workers have stressed the need to use media more concentrated than usual with membrane filters (e.g. Clark & Kabler, 1952; McKee *et al.* 1953; Taylor, Burman & Oliver, 1955), so it was decided to test whether an increase in the concentration of growth materials would improve the counts of treated cells on membrane filters. Counts of treated suspensions of strain A were done on media containing 32, 40 and 48 g/l. of 'Oxoid' CM15 granules, i.e. respectively 2, 2·5 and 3 times the concentration used in the standard nutrient broth. Also some tests were made in which counts on these media were compared with those on standard nutrient broth.

Table 4. *The effect of concentration of the medium on the viable counts of treated* E. coli *strain A on membrane filters and nutrient agar*

Trial no.	Mean viable count/drop with 'Oxoid' CM15 granules at							
	16 g/l.		32 g/l.		40 g/l.		48 g/l.	
	N.A.*	M.F.*	N.A.	M.F.	N.A.	M.F.	N.A.	M.F.
1	—	—	158·0	74·6	173·6	—	160·8	93·4
2	—	—	181·0	101·8	177·6	81·3	176·6	—
3	—	—	117·9	68·1	124·1	69·2	117·3	62·8
4	—	—	125·5	94·0	149·8	119·4	145·2	62·3
5	—	—	100·2	49·0	111·4	96·4	141·0	49·5
6	—	—	7·4	0·3	12·9	0·6	12·6	0·7
7	—	—	63·6	31·3	68·2	30·5	75·2	21·3
8	—	—	40·3	16·1	43·5	25·4	40·4	19·5
9	89·6	38·0	98·9	76·6	—	—	—	—
10	58·8	22·4	78·8	56·8	86·4	52·0	—	—
11	18·5	7·3	21·0	12·5	40·4	18·8	31·5	21·2
12	22·5	7·9	30·0	14·4	37·2	30·1	38·0	18·7
Mean response (% of N.A. count at 32 g/l.)	82	31	100	53	124	67	122	52
Proportional count on M.F.	0·39		0·53		0·57		0·43	

* N.A., nutrient agar; M.F., membrane filter.

Table 4 gives the mean viable counts under the various conditions. To indicate the general trend of the results the mean responses are given: they are expressed as percentages of the count on nutrient agar at a concentration of 32 g/l. Also, the counts on the membrane filters are expressed as a fraction of the count on nutrient agar at the same medium concentration.

It is clear that the concentration of nutrients had little effect on the magnitude of the counts on the filters relative to those on nutrient agar. Except at 16 g/l., where the results were few and not statistically conclusive, the counts on the membrane filters were significantly less than the counts on nutrient agar at the same medium concentration ($P < 0{\cdot}01$ in all cases) and this confirms the previous results at 16 g/l. (Tables 1 and 2).

Considering the membrane filters only, the mean count at 40 g/l. was somewhat higher than at the other concentrations but the differences were not statistically significant. The tendency for the counts at 48 g/l. to be low may have been caused in part by difficulties in counting because of spreading colonies. Concentrated media tend to give confluent growth and at 48 g/l. the volume of medium added to the pad was reduced slightly to minimize this effect.

The mean counts on nutrient agar were higher at 40 and 48 g/l. than at the other concentrations and the '*t*'-test indicated that these differences were significant at the 5% level.

The uptake of phenol by membrane filters

It seemed possible that adsorption of phenol on the membrane filters might play some part in the production of the low counts observed on them. Although the amount of phenol transferred to the membrane filters was minute (0·171 ml of a 0·0001% w/v solution) the whole of the phenol in a drop might have been adsorbed in the immediate environment of the treated cells, and such cells might be more susceptible to traces of phenol than untreated ones.

Quantities of 10 ml of nutrient broth containing 0·001 and 0·0001% (w/v) phenol were incubated in contact with two membrane filters for 24 hr at 37° (this is the ratio of membrane to medium used in counting). The residual phenol was determined by the indophenol method as used by Jacobs & Harris (1954). At these concentrations the method is nearly at the limit of its sensitivity, so the indophenol was concentrated by a single extraction into 1·5 ml of carbon tetrachloride. Absorption measurements were made at 565 mμ with a Unicam SP500 spectrophotometer using 2 mm cells. Under these conditions Beer's law was obeyed over the whole range of concentrations used. The results showed that the uptake of phenol was similar from both solutions: the membrane filters removed 61 and 46% (mean, 52%) of the phenol in two trials from the 0·001% (w/v) solution and 57 and 47% (mean, 53%) from the 0·0001% (w/v) solution. This may indicate that the phenol was partitioned between the nutrient broth and the non-polar membrane filter rather than being adsorbed.

To decide whether the phenol was localized on the filters near the colonies, drops of 0·1% (w/v) phenol were placed on membrane filters as usual and after incubation the latter were tested for the presence of phenol by applying the reagents directly to them. Visual inspection indicated that the phenol was distributed uniformly throughout the filter.

To confirm that the amount of phenol on the filter did not influence the counts, treated suspensions were diluted as usual and the counts were compared with those from the same suspension diluted finally into 10 times the concentration of phenol

normally present by dilution of the reaction mixture. In four trials the mean count from the higher phenol concentration was 92% of that in the normal phenol concentration: this difference was not significant.

Discussion

Although many workers (*loc. cit.*) have reported good agreement between counts on membrane filters and conventional counting methods this is not the case with *E. coli* damaged by phenol. Damaged cells may be expected to occur in water as a result of attrition or antiseptic action and it is possible that such cells may behave similarly to those damaged by phenol. This may account for the relatively poor performance of membrane filters reported by some workers (Shipe & Fields, 1954; Shipe & Cameron, 1954).

The low counts on membrane filters may be due to several causes: the filter may act as a barrier to the diffusion of nutrients especially important to the recovery of damaged cells; or the nutrient broth, the filter and the filter paper pad may contain substances toxic to damaged cells. Jacobs & Harris (1953) have shown that such substances occur in broth constituents, but it is difficult to see how these could have influenced the results since they were present equally in the control medium.

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References

Buttiaux, R., Muchemble, G. & Leurs, T. (1953). La colimetrie de l'eau sur membranes filtrantes. *Ann. Inst. Pasteur* **84**, 1010.

Clark, H. F. & Kabler, P. W. (1952). The membrane filter in water quality tests. *Amer. J. publ. Hlth* **42**, 385.

Cook, A. M. & Yousef, R. T. (1953). An improved pipette for measuring small volumes of bacterial suspensions. *J. Pharm. Pharmacol.* **5**, 141.

Goetz, A. & Tsuneishi, N. (1951). Application of molecular filter membranes to the bacteriological analysis of water. *J. Amer. Wat. Wks Ass.* **43**, 943.

Gohar, M. A. & Eissa, A. A. (1957). The use of membrane filters in the bacteriological examination of water and air. *Z. Hyg. InfektKr.* **143**, 364.

Hope, M. C. & Neill, A. H. (1956). The use of the membrane filter technique for testing water supplies in the field. *Publ. Hlth Rep., Wash.*, **71**, 1093.

Jacobs, S. E. & Harris, N. D. (1953). The presence in peptone and meat-extract of growth inhibitors active against bacteria damaged by phenol and cresols. *Atti Int. Congr. Microbiol., Rome*, **1**, 603.

Jacobs, S. E. & Harris, N. D. (1954). The value of adsorbents for detecting survival in bacterial populations exposed to phenols. *J. Pharm. Pharmacol.* **6**, 877.

Kabler, P. (1954). Water examinations by membrane filter and most probable number procedures. *Amer. J. publ. Hlth* **44**, 379.

McKee, J. E., Derby, R. L., Goetz, A., Noble, R. E. & Streicher, L. (1953). Technique of bacterial examination of water with molecular filter membranes. *J. Amer. Wat. Wks Ass.* **45**, 1196.

Miles, A. A. & Misra, S. S. (1938). The estimation of the bactericidal power of blood. *J. Hyg., Camb.*, **38**, 732.

Portner, D. M., Mayo, E. C. & Kaye, S. (1954). The evaluation of disinfectant solutions. II. Use of the membrane filter for evaluating formaldehyde solutions. *Bact. Proc.* 35.

Powers, E. L., Ehret, C. F. & Bannon, A. (1957). The membrane filter technique in radiation studies of spores of *Bacillus megaterium*. *Appl. Microbiol.* **5**, 61.

Shipe, E. L. Jr. & Cameron, G. M. (1954). A comparison of the membrane filter with the most probable number method for coliform determinations from several waters. *Appl. Microbiol.* **2**, 85.

Shipe, E. L. Jr. & Fields, A. (1954). A comparison of the molecular filtration technique with agar plate counting for enumeration of *Escherichia coli* in various aqueous concentrations of zinc and copper sulfates. *Appl. Microbiol.* **2**, 382.

Slanetz, L. W. & Bartley, C. H. (1957). Numbers of enterococci in water, sewage, and feces determined by the membrane filter technique with an improved medium. *J. Bact.* **74**, 591.

Slanetz, L. W., Bent, D. F. & Bartley, C. H. (1955). Use of the membrane filter technique to enumerate enterococci in water. *Publ. Hlth Rep., Wash.*, **70**, 67.

Taylor, E. W., Burman, N. P. & Oliver, C. W. (1955). Membrane filtration technique applied to the routine bacteriological examination of water. *J. Instn Wat. Engrs* **9**, 248.

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YEASTS CAUSING SPOILAGE IN ACETIC ACID PRESERVES

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SUMMARY: Forty-seven cultures of yeasts, isolated from 47 packs of spoiled acetic acid preserves including 11 varieties and originating from nineteen manufacturers, have been identified according to the system of Lodder & Kreger-van Rij. Forty-three cultures had the characteristics of *Saccharomyces acidifaciens* and four those of *Pichia membranaefaciens*.

Although the microbiology of the various brining processes used in the preparation of pickles has been well studied and the major flora identified, the micro-organisms causing spoilage of the finished product appear to have received little attention. A knowledge of the species responsible is of value when considering the principles upon which the keeping quality of acetic acid preserves depend. The need for a better understanding of this subject was emphasized with the introduction of pasteurized pickles in this country, and it was therefore decided to isolate and identify the micro-organisms concerned.

The few references which are to be found to the identity of the spoilage micro-organisms of pickles and sauces are confined to the American literature. Williams & Mrak (1949), while investigating an outbreak of spoilage in salad dressing, isolated a yeast which they considered had characteristics similar to *Zygosaccharomyces globiformis*. Fabian & Wethington (1950) identified yeasts from three samples of gassing salad dressing as belonging to the genus *Zygosaccharomyces* but did not determine the species. In studies on 35 cultures of yeasts isolated from 15 samples of spoiled sweet-cucumber pickles from three manufacturers, Bell & Etchells (1952) found that they were all closely related to *Z. globiformis*. Cells of one of these cultures are illustrated in a later paper by Etchells, Bell & Jones (1953).

For the present study, micro-organisms causing spoilage were isolated from pickles and sauces obtained from manufacturers in various parts of the country. Both yeasts and lactobacilli were responsible but only the former, which were found four times as often as the latter, are considered in this paper.

Materials and Methods

Origin and types of spoiled preserves. From the pickles and sauces received certain packs were selected for examination on the basis of avoiding repetitions of the same type of preserve from the same manufacturer, unless it was thought that two like packs were spoiled by different micro-organisms. All the samples thus selected had an acetic acid content of not less than 2% (w/w) and none contained benzoic acid as a preservative. Viable micro-organisms were not present in all the samples examined but, in addition to those yielding lactobacilli, 47 packs provided yeasts.

These originated from nineteen manufacturers and were as follows: pickled onions, 2; gherkins, 4; mixed clear pickles, 7; pickled red beetroot, 3; pickled red cabbage, 1; sweet chopped pickles in sauce (i.e. chutneys, etc.), 10; piccalilli, 8; tomato ketchup, 3; brown fruit sauces, 5; mint sauce, 1; salad cream or mayonnaise, 3.

Form of spoilage. Yeast spoilage was apparent as gas formation, with musty, sulphurous, or yeast-like off-odours. In clear pickles a cream or brown sediment was present and cream coloured colonies covered vegetables protruding above the surface of the liquor. In more viscous products a layer of cream to brown coloured yeast covered or partially covered the surface. In a few packs gassing was not evident.

Isolation of the spoilage micro-organisms. The spoiled preserve or a dilution thereof was plated out on a medium consisting of malt extract, 15 g; yeast extract, 2 g; peptone, 3 g; agar, 25 g; acetic acid, 15 g/litre (pH 3·25). Plates were incubated for 14 days at 25° and a single colony transferred to a similar medium without agar. After an equivalent incubation period this liquid culture was replated and another single colony grown in liquid medium. Duplicate cultures were then maintained on slopes of the above medium at 5° and in the above liquid medium at 20°. Subcultures were made at four-monthly intervals.

By isolating and maintaining cultures in the presence of 1·5% acetic acid (w/v) it was assured that only pickle spoilage micro-organisms were selected and that their acid tolerance or resistance was not lost. Sodium chloride was not included in the formula for, although it is a constituent of all acetic acid preserves, it has been found at these laboratories to have no significant preservative effect on these spoilage micro-organisms, at normally occurring concentrations. Since the colonies resulting from any one pack always appeared similar, no more than one culture from each of the 47 packs was identified.

Method of identification. The methods and system of classification of Lodder & Kreger-van Rij (1952) were followed throughout except for the use of Wickerham's non-synthetic medium (Wickerham, 1951) for observations of morphology.

Results

Forty-three cultures conformed to the standard description of *Saccharomyces acidifaciens*, with the following observations on variable characteristics.

Cell sizes ranged between 3–8×4–11·5 μ in liquid malt extract and between 3–8×3–11 μ on malt extract agar. Pseudomycelium was not formed by any culture. Sporulation was found to be good on carrot and potato wedges but poor on sodium acetate and Gorodkowa's agar. Conjugation was observed in all cultures but one.

Four cultures conformed to the standard description of *Pichia membranaefaciens*, with the following observations on variable characteristics.

Cell sizes ranged between 3–5·5×4–8·5 μ in liquid malt extract and between 3–5×5–11 μ on malt extract agar. Conjugation was observed in two cultures.

These four cultures were taken from spoiled gherkins, pickled red beetroot and piccalilli.

Tests of ability to use ethanol as the sole source of carbon and to split arbutin were not carried out during this work.

Discussion

This study demonstrates the limited range of species of yeast involved in the spoilage of British acetic acid preserves. *Saccharomyces acidifaciens* is the primary agent of spoilage, with *Pichia membranaefaciens* responsible occasionally.

The American references previously mentioned implicate *Z. globiformis* as the agent of spoilage. Lodder & Kreger-van Rij (1952) do not accept the genus *Zygosaccharomyces* and in their system this yeast is classified as *Saccharomyces rosei*. Considering the number and variety of pickles and sauces examined in this study it is somewhat surprising that, if *S. rosei* is capable of causing spoilage in these products, no cultures of this species were found among the samples examined. Only Williams & Mrak (1949) described their yeast and it differs on two points from the description of *S. rosei*. First, sucrose is only weakly, instead of strongly, fermented and assimilated, and secondly the cell is larger and more elongated than that of *S. rosei*, i.e. 4·5–6·5×5·3–8·8 μ against 2–6×2–6·9 μ. Similarly the pictures of the yeast isolated by Bell & Etchells (1952) shows oval cells, rather than the round cells typical of *S. rosei*. On this evidence alone it is impossible to be sure that these previously-examined spoilage yeasts have been wrongly classified, but it seems possible that they more closely resemble *S. acidifaciens* than *S. rosei*. Unfortunately an inulin fermentation test, which would have clarified the issue, was not reported. There would thus appear to be some doubt as to whether or not *S. rosei* is capable of spoiling acetic acid preserves; but in conclusion it is interesting to note that of the eleven strains of this yeast examined by Lodder & Kreger-van Rij none is said to have originated from products containing acetic acid, while three of the five strains of *S. acidifaciens* originated from souring wine, vinegar and salad cream respectively, all of which would contain the acid.

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References

Bell, T. A. & Etchells, J. L. (1952). Sugar and acid tolerance of spoilage yeasts from sweet-cucumber pickles. *Food Tech., Champaign*, **6**, 468.

Etchells, J. L., Bell, T. A. & Jones, I. D. (1953). Morphology and pigmentation of certain yeasts from brines and the cucumber plant. *Farlowia* **4**, 265.

Fabian, F. W. & Wethington, M. C. (1950). Spoilage in salad and french dressing due to yeasts. *Food Res.* **15**, 135.

Lodder, J. & Kreger-van Rij, N. J. W. (1952). *The Yeasts: A Taxonomic Study*. 1st ed. Amsterdam: North Holland Publishing Co.

Wickerham, L. J. (1951). Taxonomy of yeasts. *Tech. Bull. U.S. Dep. Agric.* no. 1029, p. 2.

Williams, O. B. & Mrak, E. M. (1949). An interesting outbreak of yeast spoilage in salad dressing. *Fruit Prod. J.* **28**, 141.

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THE UPTAKE OF MERCURY BY SEEDS TREATED WITH MERCURIC CHLORIDE SOLUTION, AND ITS SUBSEQUENT RELEASE

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SUMMARY: When sweet pea seeds were soaked in mercuric chloride solution a considerable quantity of mercury was taken up and was not removable by a washing procedure even more thorough than that usually employed in the technique for the surface sterilization of plant tissues. To remove the bulk of it, soaking the seed for 48 hr was needed.

Mercuric chloride is widely used for the surface sterilization of plant tissue, being highly active and requiring only a short contact period. Normally, washing with sterile water follows the treatment, but there is evidence that this does not effect complete removal. For example, Hofer & Hamilton (1937), in greenhouse experiments with *Rhizobium* and leguminous plants, found that mercurial sterilizing agents reduced the degree of nodulation; they attributed this to mercury left in the seeds. Long ago, too, Dr. H. G. Thornton (private communication) observed large zones of inhibition around mercury-treated seeds which had been planted on agar seeded with *Rhizobium*. Residual mercury may also affect seed germination (White, 1938; Ash & Allen, 1948) and may affect the vigour of the emerging seedlings (Ahmed, unpublished work). Surface sterilization of diseased wheat stem tissue with mercuric chloride may also prejudice the isolation of *Ophiobolus graminis* (Davies, 1935). However, notwithstanding these frequent indications that mercury may be retained by plant tissue treated with mercuric chloride and subsequently washed in water, there is no precise information about the extent of this retention or the subsequent rate of release. During the course of an investigation for which sterilized seeds free from retained sterilizing agent were required (Dadd, 1953), an attempt was made to assess the amount of the active agent retained by sweet pea seeds, and the results are presented below.

Experiments and Results

Two batches of 10 sweet pea seeds (variety Powerscourt) were surface sterilized in 0·2% (w/v) aqueous mercuric chloride solution for 15 min. In one case this was followed by three two-minute washings in sterile distilled water and in the other by ten such washings. A control batch of 10 seeds received ten sterile distilled water washings only. The seeds were then deposited on soil extract mannitol agar seeded with *Corynebacterium fascians*, three to each Petri dish, and incubated for 3 days at 25°. Wide zones of inhibition were observed around the treated seeds, irrespective of the number of washings. The mean zone diameters were: treated seeds, 3 washings,

41·4 mm; treated seeds, 10 washings, 40·5 mm; untreated seeds, 10 washings, 8·6 mm. Of the last, the contact between seed and agar occupied *c.* 4 mm.

Similar results were obtained with maize seeds, and evidently much mercury was retained after sterilization, even though many more washings than normal had been given.

Some idea of the difficulty of removing the absorbed mercury was afforded by an experiment in which two lots each of 50 seeds were treated for 15 min in 0·2% $HgCl_2$, washed ten times in sterile distilled water and then transferred to 20 ml of sterile distilled water. After the seeds had soaked in this in the refrigerator for 24 hr the fluid was poured off and the seeds drained. Five seeds from one lot were then planted on soil extract mannitol agar seeded with *C. fascians* and the mercuric chloride in the fluid from the other lot tested for by the cup plate method, using similarly seeded agar. Another 20 ml of sterile distilled water was then poured on to each lot of seeds, and the process repeated. In all, there were three successive 24 hr periods of soaking, followed by one two-day and one three-day period. The zone diameters around the seeds and cups were recorded after the plates had been incubated for 3 days at 25°, and the results are given in Table 1. This shows that though much of the mercury taken up by the seeds was still present after 24 hr soaking, most of that which then remained diffused out during the second 24 hr. However, a little mercury was still retained and was subsequently slowly released.

Table 1. *The release of mercury from sweet pea seeds treated for 15 min with 0·2% (w/v) mercuric chloride solution, during soaking in distilled water*

Period of soaking	Mean zone diameter (mm)*	
	Seeds†	Fluid‡
Unsoaked	40·5	—
0–24 hr	23·4	28·4
24–28 hr	9·1	14·8
48–72 hr	5·9	11·1
72–120 hr	4·6	9·0
120–192 hr	4·0	8·9

* Means of 5 replicates. † Seeds placed on soil extract mannitol agar seeded with *C. fascians*. Mean diameter of contact area, 4·0 mm.
‡ Assayed with *C. fascians*, using 8 mm. cups.

Attempts were made to estimate this uptake and release of mercury quantitatively by the cup plate method, but the results were very irregular, particularly with weak solutions, and a satisfactory standard curve could not be constructed. The dithizone colourimetric method was therefore used. Ten successive lots of 20 sweet pea seeds were treated in the same 10 ml quantity of 0·001% (w/v) mercuric chloride solution, each lot for 15 min. After treatment the seeds were taken out one by one with forceps, to avoid undue removal of fluid. The used mercuric chloride solution was retained. Each lot of seeds was given ten two-minute washings in 10 ml quantities of sterile distilled water, and all the washings were combined. The 200 seeds were collected together, soaked in 50 ml of distilled water in the refrigerator for 24 hr, the fluid

removed, replaced by 50 ml of fresh water, and so on till there had been five 24 hr soaking periods. The five lots of 'soak water' were combined and concentrated to 10 ml by distillation under reduced pressure. The combined wash waters were also concentrated to 10 ml and the mercury in these two concentrates, as well as that remaining in the original solution, was then estimated. Very little mercury was not accounted for, and the balance sheet obtained is set out in Table 2. It will be seen that over half the 100 μg of mercury applied to the seeds was absorbed by them. Of this, less than 3% was removed by the more than usually thorough washing process. The remainder was removed by the five 24 hr periods of soaking, and judging by the results given in Table 1 most of it was probably released in the first two periods.

Table 2. *The mercury balance sheet for a treatment of* 10 *lots of* 20 *sweet pea seeds in the same* 10 *ml of a solution containing* 10μg $HgCl_2$/*ml*

	Quantities of mercury (μg)		
Uptake		Release	
In original solution	100·0	Removed by ten 2-min washings	1·6
In solution after use	43·7	Removed by five 24 hr soakings	53·8
		Not accounted for	0·9
Retained by seeds	56·3	Total	56·3

Two other surface sterilization agents were examined, silver nitrate and chloramine-T. Silver nitrate was applied in 0·1% (w/v) concentration for 1 min, the fluid decanted, and the seeds washed three times for 2 min in sterile distilled water, once in 1·0% (w/v) sodium chloride and finally once more in sterile water. Chloramine-T, 1·0% (w/v), was applied for 15 min with four washings in sterile distilled water. The seeds, 200 for each treatment, were examined for inhibition zone production as described above. The silver nitrate treated seeds gave a mean zone of 15·4 mm, larger than that from untreated (washed) control seeds, where it was 8·6 mm, showing that silver was absorbed like mercury. However, the mean zone from the chloramine-T treated seeds was only 8·7 mm, indicating that removal of that sterilizing agent was easily accomplished.

References

Ash, C. G. & Allen, O. N. (1948). A comparison of methods recommended for the surface sterilization of leguminous seed. *Proc. Soil Sci. Soc. Amer.* **13**, 279.

Dadd, A. H. (1953). Infection by *Corynebacterium fascians*: antibiotic and stimulatory factors. Thesis, University of London.

Davies, F. R. (1953). The superiority of silver nitrate over mercuric chloride for surface sterilization in the isolation of *Ophiobolus graminis* Sacc. *Canad. J. Res.* C, **13**, 168.

Hofer, A. W. & Hamilton, H. C. (1937). Seed 'sterilization'. *Proc. Soil Sci. Soc. Amer.* **2**, 253.

White, H. L. (1938). The sterilization of lettuce seed. *Ann. appl. Biol.* **25**, 767.

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Donovan, K. O. (1958). *J. appl. Bact.* **21** (1), 100–103.

A SELECTIVE MEDIUM FOR *BACILLUS CEREUS* IN MILK

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SUMMARY: A medium is described by means of which cells of *Bacillus cereus* in milk may be estimated without the prior use of a heat treatment. Into a nutrient agar are incorporated: egg yolk, to demonstrate production of the characteristic opacity; citrate, to render the inoculated medium more transparent; lithium chloride and polymyxin, to reduce the growth of other bacteria. A cover of water agar limits the development of certain spreading organisms. The medium has proved of value in a survey of samples of raw milk, providing information additional to that obtained by plating after a heat treatment.

As a preliminary requirement in an inquiry into the occurrence of *Bacillus cereus* in milk and on dairy equipment, a method of quantitative estimation of both spores and vegetative cells seemed desirable. The method which Stone & Rowlands (1952) used in identifying *B. cereus* as the causative organism of 'bitty' cream is qualitative and involves a heat treatment sufficient to destroy vegetative cells. Thus, it was unsuitable for the present purpose. Similarly, the technique of Burton *et al.* (1953) for detecting small numbers of spore formers in milk, although quantitative, was unsuitable for estimating vegetative cells because of the heat treatment involved.

The most satisfactory method seemed to be a direct plating technique, capable of revealing as few as one or two cells in 1 ml of raw milk. The problem, therefore, consisted in the development of a medium in which colonies of *B. cereus* could be clearly distinguished and in which interference from other milk organisms was at a minimum. For the first purpose, a useful property of the bacillus seemed to be its ability to produce opacity in egg yolk agar (Colmer, 1948; McGaughey & Chu, 1948). For the second purpose, it seemed likely that inhibitors would be necessary, for a milk inoculum of 1 ml/plate of medium would almost certainly provide sufficient nutrients for the growth of most milk organisms.

Experimental

The basal medium. A basal medium having the following composition was selected: peptone, 5 g; beef extract (Lab-Lemco), 5 g; agar, 15 g; water, 1,000 ml; pH 7. At the time of pouring plates, egg yolk was added as a 1:1 dilution in normal saline to give a final concentration of 2·5% (v/v) of yolk. The yolk saline was not sterilized; it was prepared aseptically and held in the refrigerator. Its sterility was checked from time to time.

This medium was preferred to others tried because, in addition to giving good growth of *B. cereus*, the yolk reaction was clearly distinguishable in the presence of an inoculum of 1 ml of milk. The ease of differentiating the yolk reaction was

further enhanced by the addition to the medium of 0·5% (w/v) of tri-sodium citrate, a supplement which renders yolk and milk more transparent.

Incubation conditions. After testing different temperatures, it was concluded that 18 hr at 30° was in practice the best incubation for promoting the growth of *B. cereus* relative to that of other organisms.

The selective medium (yolk inhibitor agar). It was necessary to incorporate into the basal medium inhibitors for both Gram-negative and Gram-positive organisms. Various combinations were tried, of which polymyxin-penicillin and polymyxin-lithium chloride showed promise. The use of lithium as an inhibitor of lactic acid bacteria was suggested by the work of Macleod (1951, 1954). The combination polymyxin-penicillin was subsequently discarded because a synergistic effect against *B. cereus* was suspected. Polymyxin-lithium chloride, while not completely inhibiting the growth of all other organisms, had the effect of reducing it sufficiently to allow *B. cereus* to grow without apparent interference and to be readily detected. In occasional milk samples, Gram-negative spreading organisms, presumed to belong to the genus *Proteus* since they were not inhibited by the concentrations of polymyxin used, caused interference. This difficulty was overcome by the use of a cover of water agar over the surface of the poured plate; the spreading organisms were thereby induced to form discrete colonies.

The medium finally adopted consisted of the basal medium with the addition of tri-sodium citrate, 0·5% (w/v); lithium chloride, dried, 0·5% (w/v); polymyxin B sulphate, 50 i u/ml. After the medium in each plate had solidified, 5 ml of water agar (agar, 15 g; water, 1,000 ml; pH 7) were poured over the surface.

Results

Tests with pure cultures of B. cereus *in sterile milk.* Eleven strains of *B. cereus* from milk were used. The results are given in Table 1. It can be seen that counts of all strains were comparable with and without inhibitors. With some strains, however, there was a slight reduction in colony size in the presence of the inhibitors.

Table 1. *The effect of inhibitors in yolk agar on the growth of pure cultures of* B. cereus

Strain	Yolk agar			Yolk inhibitor agar		
	Poured plates	Spot inoculated plates		Poured plates	Spot inoculated plates	
	Colony count (/ml)	Colony diam. (mm)	Zone diam. (mm)	Colony count (/ml)	Colony diam. (mm)	Zone diam. (mm)
C.01	117	15	28	117	10	28
C.03	56	10	28	48	10	28
C.04	103	10	30	76	10	28
C.05	114	23	35	169	8	28
C.06	160	10	30	117	10	23
C.10	93	20	30	79	18	30
C.11	88	13	28	91	15	30
C.12	161	13	28	118	10	28
C.13	352	13	28	401	13	28
C.14	189	13	30	195	13	30
C.15	183	13	30	209	10	25
Mean	147	14	30	147	12	28

Tests with known inocula of B. cereus *in raw milk.* Six raw milk samples having high total bacterial counts were inoculated uniformly with a suspension of spores of *B. cereus*. Using the yolk inhibitor agar and the corresponding medium without inhibitors, 1 ml quantities of the samples were then plated. The results appear in Table 2. In every case, a good recovery of the organism was obtained on the selective medium, whereas in the absence of inhibitors there was no recovery.

Table 2. *The recovery of* B. cereus *added to raw milk samples by plating with yolk agar and yolk inhibitor agar*

Milk sample	Yolk agar *B. cereus*, count/ml		Yolk inhibitor agar *B. cereus*, count/ml	
	Uninoculated milk	Inoculated milk	Uninoculated milk	Inoculated milk
Sterile (control)	—	14	0	14
Raw 1	0	0	0	16
Raw 2	0	0	0	16
Raw 3	0	0	0	18
Raw 4	0	0	0	10
Raw 5	0	0	1	20
Raw 6	0	0	0	13

Tests with can rinses. Twenty-two can rinses were plated using the selective medium. Of these, 14 showed the presence of *B. cereus*, the average count of the organism being 16,000/can. When the inhibitors were absent from the plating medium, not even a single *B. cereus* was recovered from any rinse.

Tests with raw milk samples. From each of over 400 raw milk samples 1 ml volumes were plated with plain yolk agar and also with yolk inhibitor agar. The inhibitor agar plates gave 46 positive results, while the plain yolk agar plates gave none. A corresponding series of laboratory-pasteurized samples plated with plain yolk agar showed positive results in 26 cases. These included samples for which negative results were recorded when the raw milk was examined with yolk inhibitor agar, but the counts obtained were so low that this could be attributed to sampling error.

Discussion

Hitherto, little attention has been given to vegetative cells of *B. cereus* in milk. Thus, the possibility of using a direct plating method for detecting the organism in relatively large volumes of unheated milk has been neglected. The results given above indicate that it is impossible to obtain a count of *B. cereus* in the majority of raw milk samples without the use of inhibitors, where an inoculum of 1 ml of milk to 10 ml of medium is being used. The selection of a suitable combination of inhibitors presented some difficulty because of the wide range of organisms involved. However, that combination finally selected seemed to meet the requirements adequately, at least under the conditions of the tests. Although the growth of all the strains of *B. cereus* tested was satisfactory on the medium, the slight reduction

in colony size observed with some cultures suggests the possibility that susceptible strains may exist. Bearing in mind this limitation, it is felt that the special value of the medium lies in the fact that in an examination of raw milk it is capable of giving at least an estimate of vegetative cells and spores of strains of *B. cereus* that show a typical egg yolk reaction. This has been confirmed by parallel platings on raw and pasteurized subsamples. Thus, it is claimed that the medium is capable of supplying information not obtainable by existing methods.

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References

Burton, H., Akam, D. N., Thiel, C. C., Grinsted, E. & Clegg, L. F. L. (1953). Some investigations into the batch process of milk sterilization. *J. Soc. Dairy Tech.* **6**, 98.

Colmer, A. R. (1948). The action of *Bacillus cereus* and related species on the lecithin complex of egg yolk. *J. Bact.* **55**, 777.

McGaughey, C. A. & Chu, H. P. (1948). The egg-yolk reaction of aerobic sporing bacilli. *J. gen. Microbiol.* **2**, 334.

Macleod, R. A. (1951). Further mineral requirements of *Streptococcus faecalis*. *J. Bact.* **62**, 337.

Macleod, R. A. (1954). Dependence of the toxicity of cations for lactic acid bacteria on pH and incubation time. *J. Bact.* **67**, 23.

Stone, M. J. & Rowlands, A. (1952). 'Broken' or 'bitty' cream in raw and pasteurized milk. *J. Dairy Res.* **19**, 51.

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Papavassiliou, J. (1958). *J. appl. Bact.* **21** (1), 104–108.

THE RAPID IDENTIFICATION OF *ESCHERICHIA COLI* I BY THE PRODUCTION AT 44° OF BOTH INDOLE AND GAS FROM LACTOSE

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SUMMARY: The modification of the 44° test proposed by Mackenzie, Taylor & Gilbert (1948) is useful for the rapid identification of *Escherichia coli* I in water and foods. False positive tests caused by other coli-aerogenes bacteria, or by their association with other organisms, can be considered rare. Only a few *E. coli* I fail to produce indole or ferment lactose at 44°, and further confirmation for routine purposes is necessary only when the results at 44° are discordant, e.g. indole positive-lactose negative or indole negative-lactose positive.

The ability of *Escherichia coli* I to ferment glucose with production of gas at 46° has been known since the beginning of this century (Eijkman, 1904). Wilson *et al.* (1935) studied a large number of coli-aerogenes cultures and found that most of those which fermented lactose at 44° could be classified as *E. coli* I, while members of this group other than *E. coli* I generally failed to do so. By this study the 44° test was recommended as an important test for the classification of coli-aerogenes bacteria and the rapid identification of *E. coli* I.

Further studies in warm countries and with coli-aerogenes organisms isolated from the water of Lake Windermere (see Wilson & Miles, 1955) showed that under local conditions some coli-aerogenes bacteria other than *E. coli* I were able to produce gas from lactose at 44°.

Mackenzie, Taylor & Gilbert (1948) modified the original test by combining production of indole at 44° and fermentation of lactose in brilliant green bile lactose broth at 44°. The modification gave satisfactory results in England (Thomas *et al.* 1955), in South Africa (Roux & Dicker, 1954), in Germany (Seelemann, Papavassiliou & Wegener, 1956) and in France (Buttiaux, Samaille & Pierens, 1956).

The purpose of this study was to ascertain whether the test of Mackenzie, Taylor & Gilbert, which has been found satisfactory in other European countries, is reliable for Greece, where the climate is different.

The paper is divided into a study of coli-aerogenes bacteria isolated from (a) human faeces and (b) water and pasteurized milk.

Materials and Methods

Human faeces

Samples of faeces taken from a general hospital and from a clinic for children were examined from April 1956 to February 1957. Two hundred and fifty specimens were

examined as follows: a loopful of faeces was cultured in 5 ml of nutrient broth and incubated for 24 hr at 37°. Subcultures were made into three tubes of media: peptone water, MacConkey's broth and brilliant green bile lactose broth (BGB). These tubes were incubated in an electrically heated thermo-regulated water bath at 44±0·5°.

After 20–24 hr incubation at 44° the peptone water was tested for indole, and fermentation of lactose was noted in the two other tubes. These were then sub-cultured on to MacConkey's agar. After incubation for 20–24 hr at 37° colonies of different appearance were carefully selected and identified by the IMViC reactions (indole, methyl red, Voges-Proskauer and citrate tests), and by the production of H_2S, liquefaction of gelatin (at 22°), fermentation of lactose at 37° (in peptone water with 0·5% lactose and bromthymolblue as indicator), fermentation of MacConkey's broth at 44° and production of indole at 44°.

For the citrate utilization test Simmons' citrate agar was used. Acetylmethyl-carbinol was tested for by Barritt's method.

Lactose negative colonies grown on MacConkey's agar were subcultured on to slopes of agar containing phenylalanine, for the rapid identification of *Proteus* strains by the production of phenylpyruvic acid from phenylalanine (PPA test) (Buttiaux, Moriamez & Papavassiliou, 1956).

Water and pasteurized milk

From April to December, 1956, 21 sea and 34 well waters were examined in double and single strength MacConkey's broth for coli-aerogenes bacteria. During the same period 51 bottles of retail pasteurized milk were examined for the same organisms in single strength MacConkey's broth and BGB broth. The methods have already been detailed by Moutoussis & Papavassiliou (1957*a*,*b*).

With these two media 372 tubes showed gas production after incubation for 48 hr at 37°; they were further examined by the methods described above.

Results

Human faeces

Table 1 shows the incidence of different types among 285 coli-aerogenes strains isolated from 250 samples of faeces. Pure growths of *E. coli* I were found in 90% of the cultures, while the association of three types of coli-aerogenes bacteria was found only twice.

Most of the strains giving a positive MTG (Mackenzie, Taylor & Gilbert) test belonged to *E. coli* I; 5·2% only to a variant of *E. coli* I which gave a negative or weakly positive methyl-red test after 5 days incubation in glucose buffered broth incubated at 37°. A paper which appeared after this work had been completed (Buttiaux, Samaille & Pierens, 1956) showed that some strains of *E. coli* I failed to give a positive MR test at 37°, but did so at 32°. The Coliform Sub-committee of the Society for Applied Bacteriology (Report, 1949) recommended that incubation for not less than 3 days at 30° be used for the methyl red test.

In 1·6% of specimens only, strains other than *E. coli* I gave a positive MTG test;

1·2% were caused by three strains of *Citrobacter freundii* II. Finally, in one instance (0·4% of the samples of faeces) a positive MTG test was produced by the association of a paracolon strain of *E. coli* I, which produced indole at 44° but no gas, with a *Citrobacter*, which produced gas but not indole at 44°. Other associations were not found although they could have occurred, as a few strains of *Klebsiella* were able to ferment lactose at 44°, and some paracolon strains of *E. coli* I which were indole positive at 44° were isolated from tubes incubated at that temperature.

The incidence of the non-indole-producing *E. coli* II was low, being only 2 strains in 250 examinations (0·8%).

Table 1. *The characteristics of 285 strains of coli-aerogenes bacteria isolated from 250 samples of human faeces*

Type	No.	Indole at 44°	Gas at 44°	IMViC tests	H_2S	Gelatin	Lactose at 37° in 24 hr	PPA test
E. coli I	230	+	+	+ + − −	−	−	+	
E. coli I	2	+	+	+(+)− −	−	−	+	
E. coli I	11	+	+	+(−)− −	−	−	+	
E. coli I	2	+	−	+ + − −	−	−	+	
E. coli I, 37° negative	12	+	G	+ + − −	−	−	−	−
E. coli II	2	−	+	− + − −	−	−	+	
Cit. freundii I	3	−	+	− + − +	+	−	+	
Cit. freundii I	1	−	A	− + − +	+	−	+	
Cit. freundii II	3	+	+	+ + − +	+	−	+	
K. aerogenes I	10	−	+	− − + +	−	−	+	
K. aerogenes I	4	−	A	− − + +	−	−	+	
K. aerogenes I, 37° negative	2	−	G	− − + +	−	−	−	−
Irregular	1	−	−	− − − −	−	−	+	
Irregular	1	−	G	+ − − +	−	−	−	−
Proteus	1	−	G	+ + − +	+	+	−	+

(+), weak positive reaction; (−), test done after 2 and 5 days (incubation at 37°).
A, acid only produced at 44°; G, growth but no acid or gas at 44°.
PPA test, production of phenylpyruvic acid from phenylalanine.

Water and pasteurized milk

From the 372 tubes positive for gas at 37°, 206 gave a positive MTG test at 44°; 18 were positive for gas production at 44° and negative for indole at 44°; finally, 148 tubes were lactose- and indole-negative on subculture into three media (peptone water, MacConkey's broth and BGB broth) at 44°.

The coli-aerogenes organisms isolated from the 224 lactose positive tubes at 44° were identified as: *E. coli* I, 210 strains; *Klebsiella*, 10 strains; *Citrobacter*, 5 strains. Of the 210 strains of *E. coli* I, 4 failed to produce indole at 44°.

We failed to isolate *E. coli* I, after subculture on to MacConkey's agar, from the 148 tubes which were negative in MacConkey's broth and BGB broth at 44°.

Discussion and Conclusion

The MTG test proved to be suitable for the rapid identification of *E. coli* I in human faeces, water and pasteurized milk. The combined tests for indole from peptone

water at 44° and gas from lactose at 44° can be recommended for routine purposes, as a positive MTG test makes the time- and material-consuming IMViC reactions unnecessary. The full identification of coli-aerogenes organisms is of interest only when the combined results at 44° are irregular, e.g. indole positive-lactose negative, or indole negative-lactose positive at 44°. In our series of examinations of water and milk this was necessary for only 18 of 372 cultures (*c.* 5%).

The results encourage us to recommend the MTG test for general use in Greece. The inclusion of a test for indole production at 44° eliminates the possibility of false positives, as reported by some workers in warm countries, who use only lactose fermentation at 44° (quoted by Wilson & Miles, 1955).

False positives due to *Clostridium welchii* are not considered here; this organism does not produce indole, and although it ferments lactose and grows at 44° it is not able to grow in BGB broth.

The MTG test is also recommended for the rapid enumeration of *E. coli* I in water and foods and as a selective method for the enrichment of this organism. Counts of *E. coli* I are considered to be more accurate when the MTG test is done, because if *E. coli* I is present in small numbers in the tubes incubated at 37°, the plating of these tubes on to MacConkey's agar may give a negative result.

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References

Buttiaux, R., Moriamez, J. & Papavassiliou, J. (1956). Méthode simplifiée d'identification des *Enterobacteriaceae* n'attaquant pas rapidement le lactose. *Ann. Inst. Pasteur* **90**, 133.

Buttiaux, R., Samaille, J. & Pierens, Y. (1956). L'identification des *Escherichieae* des eaux. Test d'Eijkman et production d'indole à 44°C. Tests I.M.V.I.C. *Ann. Inst. Pasteur, Lille*, **8**, 137.

Eijkman, C. (1904). Die Gärungsprobe bei 46°C als Hilfsmittel bei der Trinkwasseruntersuchung. *Zbl. Bakt.* (Abt. 1 Orig.) **37**, 436.

Mackenzie, E. F. W., Taylor, E. W. & Gilbert, W. E. (1948). Recent experiences in the rapid identification of *Bacterium coli* Type I. *J. gen. Microbiol.* **2**, 197.

Moutoussis, C. & Papavassiliou, J. (1957*a*). The bacteriological examination of sea water in swimming places near Athens. *Acta microbiol. Hllen.* **2**, 32. (Greek text, summary in English).

Moutoussis, C. & Papavassiliou, J. (1957*b*). Contribution to the standardization of the bacteriological examination of pasteurized milk. *Acta microbiol. Hllen.* **2**, 81. (Greek text, summary in English).

Report (1949). Coliform Sub-Committee. *Proc. Soc. appl. Bact.* (2), 3.

Roux, P. & Dicker, M. (1954). The identification of faecal *B. coli* in water and milk supplies. *S. Afr. med. J.* **28**, 439.

Seelemann, M., Papavassiliou, J. & Wegener, K. H. (1956). Die bakteriologische Beschaffenheit des Gebrauchswassers auf dem Bauernhof und ihre Bedeutung für die Reinigung und Desinfektion der Milchgeräte. *Kieler Milchwirtschaft ForschBerichte* **8**, 263.

Thomas, S. B., Clegg, L. F. L., Cuthbert, W. A., Oxley, C. D., Scarlett, C. A. & Westwater, C. H. (1955). Studies of the determination of the *Bacterium coli* type I content of farm water supplies. *J. appl. Bact.* **18**, 9.

Wilson, G. S. & Miles, A. A. (1955). *Topley and Wilson's Principles of Bacteriology and Immunity*, p. 762, 4th ed. London: Arnold.

Wilson, G. S., Twigg, R. S., Wright, R. C., Hendry, C. B., Cowell, M. P. & Maier, I. (1935). *The Bacteriological Grading of Milk. Spec. Rep. Ser. med. Res. Coun.* no. 206. London: H.M.S.O.

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Egdell, J. W., Lomax, K. L., Adams, R. P. & Aitken, M. J. (1958). *J. appl. Bact.* **21** (1), 109–117.

BACTERIOLOGICAL STUDIES OF IMMERSION CLEANING OF MILKING EQUIPMENT ON COMMERCIAL FARMS

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SUMMARY: A study has been made of the immersion cleaning technique as applied on commercial farms. In an initial survey bacteriological results for rinses and swabs of the milking equipment were satisfactory on most farms, but on one farm the results were very disappointing. Further investigations at this farm showed that the poor results were caused partly by sporing bacteria which survived the caustic soda treatment, presumably in the spore state, and partly by non-sporing organisms, chiefly chromobacteria and micrococci.

These non-sporing organisms are found in abundance on the surface of dirty wash troughs, and under certain conditions re-contamination of the milking equipment can occur during the rinsing operation. Suggested modifications to the original immersion cleaning technique include the use of hot water for the before-milking hypochlorite rinse, brushing the equipment in the after-milking rinse to remove extraneous dirt, regular cleansing of the wash trough and the monthly cleansing of the basket and bin.

Since the pioneer work by Thiel, Clough & Clegg (1955), experience of the immersion cleaning technique as recommended by Thiel (1955) has been gained on nine commercial farms in the Bristol area of the South Western Region. A survey was commenced in December, 1955, on a pilot farm in Somerset and eight other farms were included later. The eight farms (Farms 1–8) followed the cleaning technique recommended by the National Institute for Research in Dairying (NIRD) and were not given special supervisory attention. On the original Somerset farm (Farm 9), where the results were disappointing from the beginning of the survey, modifications of the recommended technique were introduced from time to time and a study was made of the residual flora found on the equipment after the immersion treatment.

Sampling, and Testing Procedure

Bacteriological rinses were taken at weekly or fortnightly intervals from the equipment after the final cold rinse water containing 60 p/m of available chlorine. Milking machine clusters were rinsed with 500 ml of quarter-strength Ringer's solution containing 0·25 g of sodium thiosulphate, the solution being passed through the assembly twice. The can lids and their rubber gaskets were swabbed according to the standard technique (Ministry of Agriculture & Fisheries, 1943), the swab solution containing sodium thiosulphate. Normally the swabbing was confined to the surface of the gasket and lid exposed when this equipment is assembled, but occasionally separate swabs were taken of the underside of the gasket and the part of the lid on which the gasket rests.

Samples were examined within 6 hr by the plate colony count test on Yeastrel milk agar (YMA) at 30°. Tests for presumptive coli-aerogenes organisms at 30° and milk souring organisms at 22° were also made.

Results of Bacteriological Rinses and Swabs of Milking Equipment

Since coli-aerogenes and milk souring organisms were seldom found, the results of the rinse and swab samples have been summarized solely on the basis of colony counts and classified in accordance with the usual standards, namely, not more than 10^4 organisms/ft^2 of surface tested being deemed satisfactory and over 5×10^4/ft^2 unsatisfactory.

Table 1. *Summary classification of colony count test results of rinse and swab samples taken at 8 farms*

Farm	County	Samples examined (no.)	Percentage of samples		
			Sat.*	F. sat.*	Unsat.*
1	Gloucestershire	36	72	17	11
2	Dorset	24	92	4	4
3	Dorset	83	79	14	7
4	Gloucestershire	32	60	28	12
5	Gloucestershire	24	83	12	5
6	Gloucestershire	26	62	15	23
7	Somerset	94	78	16	6
8	Somerset	94	81	12	7
	Total	413	Average 77	15	8

* Sat., satisfactory; F. sat., fairly satisfactory; Unsat., unsatisfactory.

Table 2. *Summary classification of colony counts of cluster, gasket and lid samples taken at 8 farms*

Farm	Number of samples from								
	Cluster			Gasket			Lid		
	Sat.*	F. sat.*	Unsat.*	Sat.	F. sat.	Unsat.	Sat.	F. sat.	Unsat.
1	3	5	4	11	1	0	12	0	0
2	6	1	1	8	0	0	8	0	0
3	24	6	5	22	2	0	19	4	1
4	7	5	0	5	3	2	7	1	2
5	4	3	1	8	0	0	8	0	0
6	2	2	6	7	1	0	7	1	0
7	17	10	5	29	1	1	27	4	0
8	20	7	5	28	2	1	28	2	1
Totals	83	39	27	118	10	4	116	12	4
Percentages	56	26	18	89	8	3	88	9	3

* Sat., satisfactory; F. sat., fairly satisfactory; Unsat., unsatisfactory.

The results for Farms 1–8, summarized in Table 1, show that of the 413 samples taken only 8% were unsatisfactory. The colony counts of samples from Farm 6 and, to a lesser extent, Farm 4 were relatively high. At both farms the application

of the cleaning technique appeared to be good and there was no obvious factor to account for the relatively poor results.

It is of interest to compare the results from the cluster rinses with those from the swabs of the gasket and can lid. These have been summarized separately in Table 2, where it is seen that 27 (77%) of the 35 unsatisfactory samples were cluster rinses.

An attempt was made to relate the unsatisfactory results to factors such as the methods employed, condition of rubbers and age and strength of the caustic soda solution, but in no instance could any relationship be established. A high colony count was just as likely to occur with new or old rubbers, or with freshly-made or aged caustic soda solution. Often a high count for one cluster was accompanied by a very low count for another cluster rinsed on the same day, in spite of the fact that the treatment of both had been essentially similar.

The results for Farm 9 presented a different picture, being consistently very poor during the initial survey period. Consequently a study was made at this farm of the effect of modifications to the recommended cleaning technique, which includes rinsing free from caustic soda before milking, using water containing 60 p/m of available chlorine, and a cursory rinse in the same water after milking before returning the milking equipment to the immersion bin (before- and after-milking rinses). The investigations covered five periods when the following treatments were in operation:

1. Ordinary NIRD technique (initial survey).
2. Comparison of normal rubber with bactericidal rubber containing tetramethylthiuram disulphide (TMTD) using the ordinary technique.
3. Ordinary technique except that the milk contact surfaces of the equipment of one of the two units were brushed in the before-milking rinse water before sampling, the equipment of the other unit being merely rinsed.
4. As for 3 but, in addition, the outside of the cluster assembly of both units was brushed thoroughly in the after-milking rinse water before immersion in the caustic soda solution.
5. Essentially similar to 3, but the outside of the cluster assembly was brushed before immersion only if obvious dirt or dung splashes were present.

The results of these investigations, summarized in Table 3, show that less than one-third of the samples were satisfactory during period 1 when the ordinary cleaning technique was used. The cluster samples were generally worse than those of the gasket and lid.

During period 2, when the TMTD rubber was in use, the results were even worse. The use of the TMTD rubber made little difference to the classification of the results. Although the geometric mean colony count/ft^2 for the TMTD rubber cluster samples was only 3×10^4, as compared with $9{\cdot}6\times10^4$ for the normal rubber samples, the corresponding figures for the samples from the two types of gasket were 21×10^3 and 15×10^3 respectively. Because of these results, and since the useful life of the TMTD

rubber was shorter than that of normal rubber, the experiment was terminated. These results have been given in detail in a publication by Cousins *et al.* (1957).

Table 3. *Summary classification of colony count test results for Farm no.* 9

Period‡	Date and procedure	Samples examined (no.)	Percentage of samples		
			Sat.*	F. sat.*	Unsat.*
1	16.12.55 to 21.6.56 (ordinary technique)	51	30	35	35
2	25.6.56 to 18.10.56 (normal rubber)	36	16	42	42
	(TMTD† rubber)	36	11	44	45
3	11.12.56 to 19.3.57 (unbrushed unit)	47	57	17	26
	(brushed unit)**	47	70	13	17
4	20.3.57 to 16.4.57 (unbrushed unit)	16	75	25	0
	(brushed unit)**	16	100	0	0
5	17.4.57 to 16.7.57 (unbrushed unit)	48	73	21	6
	(brushed unit)**	48	92	6	2

‡ See text. * Sat., satisfactory; F. sat., fairly satisfactory; Unsat., unsatisfactory.
† TMTD, tetramethylthiuram disulphide. ** Unit brushed in the before-milking rinse.

At the start of period 3 extruded synthetic rubber liners, provided by the NIRD, were introduced; but within a fortnight the rubber began to break down and its use was abandoned, the experiment being continued with normal rubber liners. Brushing the equipment in the before-milking rinse water before sampling seems to have had some effect, since the proportions of unsatisfactory samples were 17% and 26% for the brushed and unbrushed unit samples respectively.

In considering the results for period 4, it should be borne in mind that bail milking is practised at this farm, and it is inevitable that in the course of milking the outside of the cluster assembly tends to become soiled. The degree of soiling will vary with the weather, but apart from very dry periods the equipment will always become contaminated to some extent. While the after-milking rinse, as recommended by the NIRD, will remove some of this contamination, any that has dried on the surfaces remains on the equipment when it is transferred to the caustic soda solution. On occasions the adhering residues of dirt and dung splashes could still be seen when the equipment was removed from the caustic soda bin at the next milking, the alkali having failed to remove it completely. For that reason the producer agreed, during period 4, to brush thoroughly the outside of both cluster assemblies in the after-milking rinse water and to continue to brush one unit in the before-milking rinse water. An immediate improvement followed; no samples from either unit were unsatisfactory.

The end of period 4 coincided with the onset of dry weather when the ground was firm and the cows did not carry soil on to the bail site. Thus during period 5, since the soiling of the clusters was reduced to a minimum, it was decided to discontinue brushing the outsides in the after-milking rinse water as a routine measure but to continue brushing the milk-contact surfaces of one unit in the before-milking rinse water. The results remained reasonably good, those for the unit brushed in the before-milking rinse water being better than those for the unbrushed unit.

Effect of equipment contamination on milk and cheese

During the period of the initial survey presumptive coli-aerogenes organisms were found in only 7 of the bacteriological rinses and milk-souring organisms in 32 out of a total of 345 tested. When the colony counts were small the flora consisted mainly of aerobic sporing bacilli, but the unsatisfactory samples showed a mixed flora consisting primarily of species of *Chromobacterium* (Wilson & Miles, 1955), with lesser numbers of micrococci and with aerobic sporing bacilli present to a variable extent.

To determine whether the residual contamination left on the equipment after immersion cleaning was likely to have a significant effect on the quality of the milk produced, weekly samples of morning and evening milk from a further farm (Farm 9) were taken at the creamery during the period 17th July to 24th October, 1956, when the worst rinse and swab sample results were obtained. Of the 24 milk samples examined for colony count, coli-aerogenes organisms and methylene blue reduction after storage at atmospheric shade temperature for 12–18 hr, only 2 failed to comply with the methylene blue test standard for raw designated milk. Three samples had a colony count in excess of 5×10^4/ml and 3 showed presumptive coli-aerogenes organisms in 10^{-2} ml. Bearing in mind the small proportion of unsatisfactory samples and the fact that the milk could have become contaminated during milking or by ancillary equipment that had not received the caustic soda treatment, it is reasonable to conclude that contamination from the immersion cleaned equipment did not materially influence the bacteriological quality of the milk.

Because of the predominance of chromobacteria in the rinse and swab samples, pure strains of these organisms were studied in the laboratory for their effect on milk. The reaction in litmus milk was usually negative; only 3 of 120 isolates caused slow acid coagulation. On tributyrin agar a number of isolates, varying from 0–30% with different samples, produced lipolysis. Although these organisms had no effect on raw milk stored at room temperature for 48 hr, it was thought that they might be important in cheese made from raw milk; therefore, two lipolytic strains were inoculated into vats of raw cheese milk at the Somerset Farm Institute to give a concentration of approximately $1{\cdot}5\times10^3$ cells/ml at the time of adding the starter. Control vats of milk from the same batch were made into cheese at the same time. The chromobacteria had no adverse effect on acid development in the milk or curd and when the cheese were one month old their flavour and texture did not differ from that of the control.

Control of contamination

Experience gained in the survey and in the subsequent investigations at Farm 9 indicated that the heavy bacterial contamination found in some rinse and swab samples was not the result of a gradual 'build-up' such as is commonly associated with some other methods of cleansing. Contamination was just as likely to occur when the caustic soda solution was freshly made or when the rubbers were renewed, as with old solution, or old rubbers in good repair.

The results for Farm 9 suggested that when the equipment was not brushed and rinsed free from extraneous dirt before immersion some organisms in the vegetative state, including the chromogenic flora, survived in the caustic soda and were carried

through the solution and lodged on the internal surfaces of the equipment. To investigate this possibility a spare teat-cup shell fitted with a new, extruded, stretched liner was sterilized and placed in the caustic soda solution with the normal equipment. The teat-cup was only removed from the bin at weekly intervals, when it was rinsed in the before-milking rinse water and tested by swabbing. Thirteen weekly tests were made and the colony counts ranged from $2{\cdot}5\times10^2$ to $5{\cdot}2\times10^5/ft^2$ of surface swabbed. It seemed at that time that this teat-cup had become contaminated while it was immersed in the caustic soda solution, but subsequent investigation showed that the contamination could have occurred in the rinse trough.

Since chromobacteria were the predominant contaminants in most of the samples from Farm 9, and as their presence appeared to be associated with extraneous dirt, samples of yard scrapings were examined. These were found to contain up to 2×10^8 chromogenic organisms/g.

Laboratory tests were then made to determine whether the micro-organisms survived in 2·5% caustic soda solution. Teat-cup liners and smaller strips of rubber were artificially contaminated with films of milk residues, dung, soil, and with pure cultures of *Chromobacterium* species isolated from rinse and swab cultures. After allowing the inocula to dry the rubbers were suspended in solutions of 2·5% caustic soda overnight. Although these tests were repeated several times under different temperature conditions, it was not possible to demonstrate survival in sufficiently large numbers to explain the unsatisfactory rinse and swab samples. Spores of aerobic bacilli survived regularly but only an occasional non-sporing organism was found in some of the cultures. These experiments served to show that a greater number of organisms survived when the temperature of the solution was only 5°, the survivors decreasing steadily with increase in temperature to 25° when no viable organisms remained.

Tests were then made on farms to determine the extent of the contamination of the equipment on removal from the caustic soda solution and prior to rinsing in the before-milking rinse water in the wash trough, and also the contamination surviving on the internal surface of the bin, on the basket in which the equipment is placed and in the caustic soda solution itself.

Rinse samples of equipment taken directly from the bin were neutralized before examination. Rinse samples of the same equipment were also taken after rinsing in a 60 p/m hypochlorite solution in the wash trough, which is the normal procedure prescribed in the technique. The colony counts of rinse samples from equipment before and after rinsing in the hypochlorite solution were in some instances similar, only aerobic sporing bacilli being present. In other cases the colony count of the rinse sample after the equipment had been rinsed in the hypochlorite solution was appreciably greater than that of the corresponding sample before rinsing, suggesting that some contamination had been picked up from the trough itself. Apart from two samples, both from gaskets, when some non-sporing chromogenic organisms were present, the flora of equipment tested as it was taken from the bin was confined to aerobic sporing bacilli.

In the course of this work it was observed that the bacteriological rinse technique

(whereby the Ringer's solution is passed through the cluster twice) on some occasions removed only a small proportion of the bacteria. This applied more particularly to tests of equipment direct from the bin and was clearly demonstrated by swabbing the equipment immediately after rinses had been taken.

Tests of the basket, the interior surface of the bin and the caustic soda solution revealed a variable degree of contamination, consisting almost entirely of aerobic sporing bacilli which no doubt survived the effect of the caustic soda while in the spore state. The highest colony counts were obtained at Farm 9 where a sample of the sludge which tends to accumulate at the bottom of the bin contained 4×10^2 organisms/ml and a swab of the basket $2\times10^4/ft^2$.

Contamination from wash troughs

The results of bacteriological rinses and swabs taken before and after the before-milking rinsing of the equipment suggested that some of the residual flora apart from spores may originate from the wash trough itself. Swab tests were made of troughs on a number of farms where one trough is used solely for rinsing purposes. Table 4 shows that the colony counts varied considerably and that coli-aerogenes and milk souring organisms were present in some cases. The flora was mixed, but it is significant that chromogenic organisms were invariably predominant. The highest counts were obtained at Farm 9.

Table 4. *Bacteriological analysis results for swab samples from wash troughs*

Farm	Colony count ($10^3/ft^2$)	Presumptive coli-aerogenes	Milk souring organisms
A	8·5	−	−
	7·6	−	−
	11·7	−	−
	26·0	−	−
	1·6	−	−
B	1·1	−	−
	21·0	−	−
C	0·7	−	−
	19·0	+	+
	27·0	−	−
	65·0	−	+
D	900	−	+
	295	−	+
E*	2,800	+	+
	267	−	−
	25·0	−	−
	3,200	−	+
	1,100	−	+
	440	−	−

* E is Farm no. 9.

Using a milking machine cluster sterilized by autoclaving, tests were made to ascertain the extent to which heavily contaminated wash troughs could re-contaminate equipment during rinsing. When no hypoclorite solution was added to

the final rinse water in the trough the number of organisms picked up by the cluster was *c.* $10^4/ft^2$, the highest count being $5{\cdot}3\times10^5$, but when the rinse water in the trough contained 60 p/m available chlorine and the cluster was allowed a contact time of 2 min in the solution, negligible counts were obtained.

Discussion

If a reasonably good cleaning routine is regarded as one with which not more than 15% of rinses or swabs are unsatisfactory, seven of the nine farms in the survey achieved a satisfactory standard. On the other two farms, and particularly on Farm 9, the standard attained was disappointing, emphasizing that adherence to the recommended cleaning procedure will not necessarily guarantee satisfactory bacteriological results. Evidence has been obtained at Farm 9 that when results for rinses and swabs are good the flora consists mostly, if not entirely, of the spores of aerobic bacilli which can survive the caustic soda treatment. With an adequate after-milking rinse and the removal of extraneous dirt from the outsides of the clusters by brushing it seems unlikely that the number of spores on the equipment would be excessive, provided that the surface of the equipment, particularly the rubbers, does not become rough or damaged. If this happens there is the possibility that non-sporing organisms could be protected from the action of the caustic soda.

When unsatisfactory rinse and swab results occur it has been found that the flora is not always confined to aerobic sporing bacilli; often it is mixed in character, with chromobacteria and micrococci predominating. The early investigations at Farm 9 suggested that this non-sporing flora was able to survive the effect of the caustic soda in the immersion bin because the spare milking machine liner placed in the bin and tested after rinsing in the wash trough showed extensive contamination, and brushing the equipment in the before- and after-milking rinse water produced a remarkable improvement in the results. But Table 4 shows that wash troughs used only for rinsing purposes can become heavily contaminated, and unless they are cleansed daily it has been demonstrated that this contamination can be picked up on the surfaces of milk equipment during the before-milking rinsing, especially if care is not taken to add the recommended dose of hypochlorite and if the contact time is insufficient. Therefore, the non-sporing flora found on the spare milking machine liner at Farm 9 probably originated from the wash trough surface.

The NIRD technique does not prescribe that the equipment shall be in contact with the chlorinated before-milking rinse water for a stated period of time, the object of the rinsing being merely to remove the caustic soda. The satisfactory results obtained at Farm 9 during periods 3 and 4 were probably due not only to the fact that the equipment was less heavily soiled during milking because of the prevailing dry weather, but also to the brushing of the equipment in the before- and after-milking rinse water.

Samples of rinse water taken from the trough after the before-milking rinsing of the equipment was completed, and tested after the immediate neutralization of the chlorine, still showed the presence of chromobacteria and micrococci in addition to spores. This suggests that the contamination from dirty troughs is released

progressively as rinsing proceeds and that the flora is protected to some extent by particulate matter. It is also evident that these non-sporing organisms are more tolerant of chlorine than lactic acid streptococci and coli-aerogenes organisms.

It is not contended that contaminated wash troughs are the only cause of unsatisfactory rinses and swabs, but they can be an important factor where one trough is used only for rinsing purposes and is not kept clean. This contention applies with equal force to other systems of chemical sterilization. On some farms hot water is used for the before-milking rinse solution, since it tends to prevent the slipping of rubbers from the metal connections of the milking machine; this practice is to be recommended as it has the added effect of rendering the chlorine more effective as a sterilizing agent. Nevertheless, the aim should be to keep wash troughs as clean as the milking equipment itself if the full effect of the cleansing process is to be preserved. Ideally the wash trough should be thoroughly cleansed daily with hot detergent solution containing 250 p/m of chlorine.

As unsatisfactory rinse and swab samples can be obtained from equipment tested directly after removal from the caustic soda, when the flora consists essentially of spores that have survived the alkali, control of this form of contamination is necessary. There is evidence that brushing the milk equipment during the after-milking rinsing to remove extraneous dirt and residues is important; cleansing the milk equipment, basket and bin with hot detergent-hypochlorite solution at monthly intervals when the caustic soda is changed may also help to control the contamination. It may even be necessary on some farms to give the milk equipment a hot detergent-hypochlorite wash at more frequent intervals for the same reason, and to prevent the accumulation of soapy deposits which afford protection for micro-organisms. Even with these additional precautions high colony counts of equipment may still be obtained sporadically and there is need for further investigation of the factors involved.

References

Cousins, C. M., Clegg, L. F. L., Thiel, C. C., Akam, D. N., Jones, G. E. & Egdell, J. W. (1957). Studies on the use of bactericidal rubber in milking machine clusters. *J. appl. Bact.* **20**, 158.

Ministry of Agriculture and Fisheries (1943). *The Swab Method of Examination of Milk Plant.* Form no. C.195/TPY. London: H.M.S.O.

Thiel, C. C. (1955). Immersion cleaning of direct-to-can milking equipment. *Milk Prod.* **2** (11), 12.

Thiel, C. C., Clough, P. A. & Clegg, L. F. L. (1955). Immersion cleaning of milking equipment. *J. Dairy Res.* **22**, 156.

Wilson, G. S. & Miles, A. A. (1955). Topley and Wilson's *Principles of Bacteriology and Immunity*, p. 726, 4th ed. London: Arnold.

(*Received* 24 *March*, 1958)

Ridgway, J. D. (1958). *J. appl. Bact.* **21** (1), 118–127.

THE INCIDENCE AND THERMAL RESISTANCE OF MESOPHILIC SPORES FOUND IN MILK AND RELATED ENVIRONMENTS

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SUMMARY: A spore 'spectrum' is described of aerobic mesophiles capable of resisting different heat treatments. It is shown that *B. licheniformis* is the most common spore former found in bulk milk but since its spores are rapidly destroyed at 100°, the more heat resistant *B. subtilis* is the dominant surviving spore former in commercial sterilized milk. The thermal resistance of strains of *B. subtilis* and *B. licheniformis* isolated from different sources has been investigated and the strains of *B. subtilis* typed according to the behaviour of their spores when heated at 100°. All strains of *B. licheniformis* were destroyed more rapidly by boiling for 2 min than strains of *B. subtilis* but only those strains of the latter which showed some degree of heat activation were more resistant than *B. licheniformis*. The 'resistant' and heat activated strains of *B. subtilis* appear to be sparsely distributed in nature and were only isolated from sterilized milk where the heat treatment applied would tend to eliminate other strains. The spore content of bovine faeces was similar to that in bulk milk and the total spore content varied seasonally, the spore content of faeces being on the average a hundred times greater during indoor feeding than during the period when the cattle were fed outside. A faecal infection of the milk in the ratio of $1:10^4$ would infect the milk with spores at about the same concentration as they are found in bulk raw milk, and it is suggested that bovine faeces could be a primary source of spore formers in milk supplies.

Several workers have now confirmed the seasonal variation in the number of mesophilic spores found in raw milk supplies in the United Kingdom. Though Franklin, Williams & Clegg (1956) confirmed this observation in raw milk supplies, they found no such variation in sterilized milk. It is quite probable that this is because of intentional variations of thermal treatment applied in the ten sterilizing depôts where they obtained samples. It is not unusual when atmospheric temperatures are high for operators using static tanks or autoclaves to increase the treatment conditions either by raising the temperature or prolonging the holding time. On the other hand where processing conditions are standardized and automatically controlled, this seasonal variation of the spore content of sterilized milk is readily discernible.

A comparison is made of the results obtained by Franklin *et al.* (1956), Candy & Nichols (1956) and the earlier work reported by Ridgway (1954, 1955), with new work carried out during the last three years. These results provide a 'spectrum' of both the number and types of spores found in milk and capable of resisting different time-temperature conditions from 100° for 2 min to 110° for 40 min.

Experimental and Results

Numbers of spores in milk supplies surviving different heat treatments

To facilitate comparison, experiments and data have been grouped as follows:

(a) During the period May, 1953–May, 1954, 621 tanked supplies of depôt milk

(in units of 1,500 gal) were examined at the rate of 50/month, by heating 10 ml samples to 97·5°–100° for 2 min, plating with Yeastrel milk agar (YMA) and incubating for 3 days at 30° (Ridgway, 1955). These results, expressed as spores/100 l., are given in column (a), Table 1.

(b) Franklin *et al.* (1956) examined 91 samples of bulk tanker milk from 10 different dairies during the period January December, 1955. They determined the most probable number of spores/100 ml of milk by the dilution technique following steaming of the milk for 30 min. Their results, converted to spores/100 l. of milk, are given in Table 1, column (b).

(c) Concurrently with the work described under (a), 43 samples were examined for the number of mesophilic spores in bulk milk supplies capable of resisting 105° for 15 min. The dilution technique described by Burton *et al.* (1953) was used, the heated milks being incubated for a maximum of 21 days at 23°–24°. These results are given in Table 1, column (c).

(d) Using the same technique as described in (c) above, 504 samples of bulk tanker milk were taken from seven different depôts supplying the same sterilized milk dairy during the period February, 1955–February, 1956, inclusive. The mean monthly values are given in Table 1, column (d).

Table 1. *A comparison of the monthly average spore contents of different milk supplies following various heat treatments*

Period	No. of spores/100 l. after heating at:					
	100°, 2 min (a)*	100°, 30 min (b)	105°, 15 min (c)	105°, 15 min (d)	106·5°, 35 min (e)	110°, 40 min (f)
January	7,150,000	143,700	1,500	3,300	169	14
February	7,350,000	95,300	4,000	3,000	160	14
March	6,640,000	—	5,000	3,200	164	11
April	4,130,000	29,440	3,000	1,200	140	5
May	930,000	11,410	1,200	400	61	3
June	520,000	—	500	200	28	0
July	530,000	3,800	600	100	39	0
August	250,000	3,980	700	300	41	1
September	490,000	12,310	800	200	36	1
October	970,000	20,530	1,200	200	52	3
November	3,250,000	32,130	2,000	1,600	131	8
December	4,110,000	73,550	1,200	3,400	167	14
Mean seasonal values						
Winter (Nov-April)	5,380,000	93,000	2,780	2,620	155	9
Summer (May–Oct.)	615,000	8,700	830	230	42	1

* (a) etc. Type of supply: see text for description.

(e) During the period January, 1951–December, 1952, 2,448 pint bottles of sterilized milk previously treated in continuous automatic sterilizers at 106·5° for 35 min were sampled at the rate of 24/week (Ridgway, 1954). The bottles were incubated at 23°–24° for a maximum of 21 days, when if they were still entirely satisfactory they were considered to be sterile. Based on the assumption that the

survival of only one spore would result in failure of the milk during this 21 day incubation, the survival of spores/100 l. has been calculated and recorded in Table 1, column (e).

(f) During the period January, 1952–December, 1955, a total of 3,176 pint bottles previously treated in continuous automatic sterilizers at 110°±0·5° for 40 min have been subjected to the same incubation test as described under (e). The survival of spores/100 l. of milk has again been calculated in the same manner and recorded in Table 1, column (f).

These results show that the seasonal variation in the spore content of milk supplies is obvious following widely different conditions of heating. They also show that the spore content of winter milk is on the average about ten times higher than that of summer milk.

It is important to emphasize that the laboratory incubation tests of sterilized milk described under (e) and (f) above do not necessarily indicate the actual commercial keeping quality of the milk but merely reflect its potential keeping quality. It is rare in the United Kingdom for day atmospheric temperatures to be as high as 23°–24° (75°F) when night temperatures are also above 18·5° (65°F). Such days only occur during the months of June to August when the intrinsic spore content of raw milk supplies is lowest. Thus it is, that of a further 1,593 pint bottles of sterilized milk treated as in (f) but stored for 21 days at prevailing atmospheric temperatures, only one bottle developed a bacteriological fault.

Distribution and types of aerobic mesophiles from raw milk, sterilized milk and other sources

Grinsted & Clegg (1955) confirmed that a simplified classification based on morphology, the egg yolk reaction, Voges-Proskauer test, gelatin and starch hydrolysis and anaerobic growth in a glucose broth medium, would be sufficient to identify the majority of mesophilic spores found in milk. Franklin *et al.* (1956) used the same techniques and these methods have also been used in the original work described in this paper. Candy & Nichols (1956) omitted the egg yolk reaction but otherwise used the same methods and in addition observed growth in Koser's citrate medium, 5% NaCl broth, and the fermentation of glucose and lactose in a broth containing $(NH_4)_2HPO_4$ as the sole source of nitrogen.

The results provided by all these workers are given in Table 2, together with the recent identification of isolates from sterilized milk following incubation and after development of sweet coagulation. In addition, the classification of spores found in milk sampled direct from the udder, in byre dust, and in bovine faeces is also given.

There is general agreement that the dominant mesophilic spores in milk supplies are *B. licheniformis* and *B. subtilis*, and it is also clear that while the spores of *B. licheniformis* are more common they are also the less heat resistant. It will be observed, however, that a greater proportion of *B. subtilis* spores was obtained in this work (22·7%) from milk boiled for only 2 min than in the studies of either Grinsted & Clegg (7·4%) or Franklin *et al.* (7·1%) from milk boiled for 30 min. An observation on this particular point is made later. The results also suggest that the

higher the temperature of treatment the greater will be the proportion of surviving spores of the *B. subtilis* type.

Table 2. *The findings of different workers on the distribution and types of aerobic mesophiles in different environments*

Authors, and sources of isolates		Heat treatment	No. of isolates	Percentage distribution of species						
				subtilis	*licheniformis*	*brevis*	*coagulans*	*circulans*	Other species	Not identified
Ridgway	Bulk milk	100° for 2 min	44	22·7	70·5	0·0	2·3	0·0	0·0	4·5
Grinsted & Clegg (1955)	Bulk milk	100° for 30 min	189	7·4	73·6	15·3	0·0	1·6	2·1	0·0
Franklin *et al.* (1956)	Bulk milk	100° for 30 min	424	7·1	62·3	15·1	0·0	3·1	3·9	8·5
Candy & Nichols (1956)	Sterilized milk	Not stated	206	61·0	13·0	1·0	2·0	13·0	10·0	0·0
Grinsted & Clegg (1955)	Sterilized milk	Not stated	42	78·6	4·8	0·0	0·0	16·6	0·0	0·0
Franklin *et al.* (1956)	Sterilized milk	Not stated	100	44·0	26·0	4·0	1·0	8·0	2·0	13·0
Ridgway	Sterilized milk	106·5° for 35 min*	147	81·0	15·7	2·7	0·0	0·0	0·6	0·0
Ridgway	Sterilized milk	110° for 40 min*	38	92·1	7·9	0·0	0·0	0·0	0·0	0·0
Ridgway	Udder	100° for 2 min	39	20·5	71·8	0·0	0·0	0·0	0·0	7·7
Ridgway	Byre dust	100° for 2 min	30	10·0	86·8	3·4	0·0	0·0	0·0	0·0
Ridgway	Bovine faeces	100° for 2 min	70	5·7	77·2	4·3	0·0	0·0	1·4	0·0
Ridgway	Bovine faeces	105° for 15 min	25	84·0	4·0	0·0	8·0	0·0	4·0	0·0

* Associated with sweet coagulation only.

It will be seen that the proportions of *B. licheniformis* and *B. subtilis* spores in byre dust and bovine faeces are much the same as in bulk milk. Further, it will be observed that spores isolated from bovine faeces which have survived temperatures above atmospheric boiling (i.e. 105° for 15 min) are dominantly *B. subtilis*, as in the case of sterilized milk.

Thermal resistance of cultures of B. subtilis *and* B. licheniformis *isolated from different sources*

The thermal resistances of 51 *B. subtilis* and 21 *B. licheniformis* cultures isolated from udder, bulk and sterilized milk and from bovine faeces were studied by the following method.

A well isolated colony of an already identified culture was picked off into nutrient broth and incubated at 37·5° overnight. The following morning a standard loop dilution was added to molten YMA and poured on to a plate which was then incubated for 2 days at 37·5°. A well isolated colony from this plate was added to nutrient broth which was then incubated overnight at 37·5°. The following morning 1 ml of this broth culture was added to 10 ml of hardened YMA in a 6 oz medical flat bottle which was then incubated at 37·5° for 2 days. The remainder of the broth culture was used as the inoculum for confirmatory tests to check the identity of the organism.

After 2 days, the 6 oz medical flat was removed from the incubator and 20 ml of sterile distilled water added from a 2 oz medical flat containing glass beads. The growth on the agar was washed off with the distilled water and returned to the 2 oz bottle which was then incubated overnight at 37·5°. The following morning the spore suspension was thoroughly shaken for 5 min and then filtered through sterile cotton wool. Ten ml of the filtrate were collected in a sterile $6\times\frac{5}{8}$ in. test tube, heated to 63·5° for 35 min to destroy vegetative cells, quickly cooled and immediately placed in the refrigerator at 5°. Two hours later the suspension was removed from the refrigerator for just sufficient time to extract 1 ml to determine the total spore count by plating (it was necessary to have an approximate idea of the count of the suspension to equalize subsequent inoculations in milk). The suspension was held in the refrigerator for 6 days when (dependent on the count of the suspension) 1 ml of the determined dilution of it was added to 160 ml of sterile milk in a 6 oz medical flat. It was the intention to have a spore count in the diluted milk of 10^4–10^5/ml.

The inoculated milk was shaken thoroughly for 10 min and then dispensed in 10 ml quantities in fifteen $6\times\frac{5}{8}$ in. test tubes.

Three tubes were subjected to each of the following treatments: 63·5° for 35 min; 100° for 2 min; 100° for 5 min; 100° for 15 min; 100° for 30 min. For the heating tests at 100° the stop-watch was started when the control tube reached 97·5°.

Following the above heat treatments, each tube was plated at an appropriate dilution using YMA and incubation for 3 days at 30°. The means of the triplicate determinations are expressed in Table 3 as the logs of the mean counts of surviving spores.

The *B. subtilis* cultures isolated from sterilized milk have been grouped into types A, B and C. Type A spores showed increasing heat activation up to 30 min boiling. Type B spores were heat activated by boiling for 2 min but tended to be destroyed with more prolonged boiling. Type C spores showed little or no heat activation and were rapidly destroyed after boiling for 5 min.

The mean averages of surviving spores of each type of *B. subtilis* (including the type C isolated from sources other than sterilized milk), and the mean averages of surviving spores of *B. licheniformis*, are given in Fig. 1, where it is assumed that the count at 0 min is the same as after 63·5° for 35 min.

The phenomenon of heat activation of spores is well known but it is interesting that other experiments carried out with certain of the type A cultures revealed that activation was still occurring after boiling for 60 min and in certain cases after heating at 105° for 30 min and even 110° for 10 min.

The type A, and to a lesser extent type B, spores are obviously the most difficult to destroy by heat; but it is interesting that they have so far only been isolated from sterilized milk. This is presumably because they are not widely distributed in nature and the process of sterilization would normally eliminate the less heat resistant strains, thereby tending to select strains which are more heat resistant.

B. licheniformis can usually be distinguished from all types of *B. subtilis* since a large proportion of the spores is destroyed by only 2 min boiling. On the other hand, though the spores of *B. subtilis* type C show some resistance to boiling for 2–5 min, these appear to be destroyed more rapidly at the longer periods of heating than

Table 3. *The results of thermal resistance tests on spore forming organisms from different sources*

Culture type, source and no.	Log_{10} mean count of spores surviving treatments of: 63·5°, 35 min	100°, 2 min	100°, 5 min	100°, 15 min	100°, 30 min
B. subtilis, type A, sterilized milk					
S57	3·57	4·59	4·71	5·04	5·13
S52	3·08	4·38	4·57	4·82	5·06
S53	2·00	3·11	3·00	3·29	3·17
S61	2·00	2·76	2·82	2·91	3·23
S13	3·60	4·02	4·30	4·28	4·32
S81A	4·53	5·24	5·31	5·40	5·48
S81B	4·06	4·53	4·61	4·66	4·74
S94	4·03	4·61	4·73	5·21	5·11
S20	3·86	4·09	4·26	4·32	4·58
Mean	3·42	4·15	4·26	4·43	4·53
B. subtilis, type B, sterilized milk					
S44	4·45	4·90	4·78	4·70	4·72
S62	4·60	4·69	4·85	4·80	4·63
S 2	4·48	4·66	4·69	4·54	4·54
S 7	4·41	4·80	4·71	4·66	4·66
S14	4·05	4·21	4·28	4·16	3·88
S 6	4·73	4·83	4·75	4·62	4·56
S11	5·08	5·11	4·96	4·86	4·65
S12	3·71	3·81	3·93	3·85	3·86
S37	4·48	4·91	4·91	4·84	4·69
S29	4·73	5·31	5·43	5·08	5·19
S69	5·34	5·25	5·17	5·26	5·06
S46	5·08	5·09	5·11	5·05	4·92
S86	5·09	5·08	5·10	4·96	4·85
S87	5·63	5·67	5·60	5·56	5·56
S95	5·23	5·32	5·26	5·20	5·20
S77	5·12	5·23	5·22	5·22	5·20
S78	5·45	5·43	5·48	5·46	5·41
S63	5·26	5·30	5·10	5·06	4·95
S35	4·91	4·88	4·71	4·73	4·90
Mean	4·83	4·97	4·95	4·87	4·81
B. subtilis, type C, sterilized milk					
S60	4·86	4·71	4·61	2·76	0·30
S83	5·26	5·41	5·17	1·52	0·95
S88	5·22	5·21	5·23	2·82	1·23
S91	5·27	5·16	4·94	2·65	0·36
S58	3·66	2·00	1·78	0·48	0·00
S70	5·00	4·95	4·01	1·73	0·40
S19	5·35	4·93	4·84	1·00	0·48
S28	4·58	4·04	3·56	1·30	0·43
S41	5·00	4·81	4·57	0·00	0·36
S55	5·47	5·02	3·58	1·11	0·65
Mean	4·42	4·12	3·87	1·33	0·52
B. subtilis, type C, udder					
U18	5·24	4·99	4·32	0·60	0·30
U63	4·96	4·78	4·65	1·67	0·23
U65	4·94	4·84	4·49	1·70	0·48
U74	4·84	4·59	4·63	1·63	0·52
U76	5·25	5·13	4·87	1·80	0·42
U77	5·20	5·11	4·80	1·36	0·63
U80	4·97	5·13	5·08	2·93	0·11
U64	5·40	5·11	4·50	1·72	0·97
Mean	5·10	4·97	4·66	1·68	0·46
B. subtilis, type C, bovine faeces					
F117	5·00	5·01	4·98	1·78	1·00
F121	5·03	5·04	5·07	1·08	1·04
F 83	5·06	4·97	3·80	0·36	1·48
F 85	4·40	3·27	2·03	1·53	0·36
F 82	4·73	4·56	3·60	1·84	0·00
Mean	4·84	4·57	3·90	1·33	0·48
B. licheniformis, sterilized milk					
S15	4·97	3·45	2·40	2·50	1·04
S68A	5·02	4·33	3·05	1·41	0·60
S68B	5·31	4·32	2·45	1·23	0·48
S43	4·97	3·71	2·59	1·08	0·00
S 5	4·73	3·63	2·02	0·85	0·48
S 9	5·17	3·61	2·91	1·30	0·30
Mean	5·02	3·74	2·57	1·40	0·48
B. licheniformis, udder					
U44	5·21	4·72	3·25	2·59	0·86
U61	5·22	4·26	3·23	3·00	1·96
U66	5·52	4·53	3·54	1·95	1·49
U68	4·98	4·45	2·90	2·14	1·15
U69	4·95	4·72	4·49	2·19	0·30
U70	5·57	4·86	3·96	2·30	0·78
Mean	5·25	4·58	3·56	2·36	1·09
B. licheniformis, bovine faeces					
F127	4·48	4·06	3·40	1·18	$\bar{1}$·85
F160	4·89	2·99	2·67	$\bar{1}$·69	0·30
F 82	4·73	4·56	3·60	$\bar{1}$·84	0·00
F105	4·63	3·27	2·33	1·40	1·20
F106	4·64	3·39	2·56	1·11	0·00
F109	4·74	3·54	2·78	1·48	0·30
F111	4·99	3·95	2·99	0·43	$\bar{1}$·48
F112	4·90	3·88	2·85	1·49	$\bar{1}$·48
F115	4·81	3·86	2·93	0·89	0·00
Mean	4·76	3·72	2·90	1·07	0·33

the spores of *B. licheniformis*, so that after 15 or 30 min the proportion of surviving spores is similar for both *B. subtilis* type C and *B. licheniformis*. The shape of the thermal death curve of *B. subtilis* type C may account for the fact that we obtained

a higher proportion of *B. subtilis* isolates from bulk milk boiled for only 2 min compared with the proportion found by other workers after boiling for 30 min.

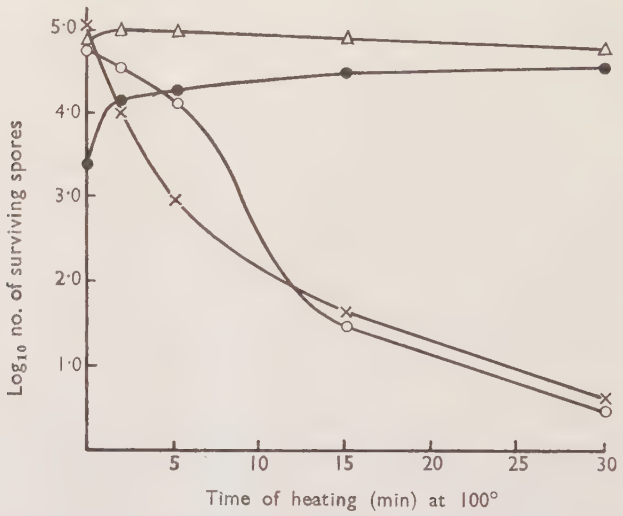

Fig. 1. The effect of heat on the activation of certain *Bacillus* spp. and strains. Crosses, *B. licheniformis*; full circles, *B. subtilis* type A; triangles, *B. subtilis* type B; open circles, *B. subtilis* type C.

The incidence of mesophilic spores in bovine faeces

In the winter of 1954, visits to a producer whose milk consistently gave high spore counts during the winter months revealed that milk taken direct from the udders of his cows was quite heavily infected with spores (Ridgway, 1955). Visits were continued during the spring to cover the period when the cattle were being turned out to grass for the first time. On each visit the same three cows were milked. The udders were carefully washed with a dilute hypochlorite solution, the first three or four streams of milk discarded and then 200–250 ml of milk from each of the three cows collected directly into sterile plasma bottles. A milking machine unit was rinsed with sterile milk and then used to milk the first of the three cows. A sample was then taken from the unit and a further milk sample taken from a can after milking the entire herd. Finally, samples were taken of bovine faeces (direct from the cow) and of the dust in the byre.

In every case the spore count was determined after heating the milk at 100° for 2 min (milk suspensions of the faeces and the dust were used, employing a technique described below). The results given in Table 4 are the mean values of the samples taken during the seven weeks of visiting. They indicate a marked reduction in the spore content of bovine faeces when the cattle discontinued indoor feeding. This also occurred in all the milk samples. There was a slight reduction in the spore content of the byre dust but it was not considered significant.

Unfortunately it was not until the early spring of 1957 that it was found possible to explore further the fluctuation in the spore content of faeces, but during the period

March 1957–February 1958, 23 visits were made to the producer to collect faeces direct from the cow. The morning following the visit to the farm a pint sample of herd milk was taken at the dairy.

Table 4. *The effect of the change from indoor to outdoor feeding on the spore content of a producer's milk supplies, bovine faeces and byre dust*

Housing of animals and dates	Mean spore count					
	Milk from			Rinse of milking machine (1 unit)	Faeces (1 mg)	Byre dust (1 mg)
	Udder (1 ml)	Machine after first cow (1 ml)	Can after whole herd (1 ml)			
Cows indoors (30th March, 6th & 13th April)	66	110	210	1,500	2,150	1,890
Cows in at night only (20th & 24th April)	23	20	51	250	640	1,460
Cows out (4th & 11th May)	3	10	8	250	100	1,320

Table 5. *The total bacterial count and spore content of bovine faeces and a producer's milk supplies during an eleven month period*

Date	Faeces (counts/mg)					Milk (counts/ml)			
	Total count (10^3)	Spores surviving				Total count (10^3)	Spores surviving		
		63·5°, 35 min	100°, 2 min	100°, 15 min	105°, 15 min		63·5°, 35 min	100°, 2 min	100°, 15 min
Cows indoors									
14.3.57	65	12,600	6,000	171	0·31	17	125	95	18
21.3.57	72	25,000	12,000	700	0·19	50	470	340	12
28.3.47	7	1,300	780	19	0·08	109	145	145	12
4.4.57	143	11,800	2,400	25	0·22		Not sampled		
11.4.57	59	7,500	2,000	360	0·22	4	280	140	12
Cows turned out to grass 16.4.57									
18.4.57	20	500	125	1	<0·01	5	250	215	20
25.4.57	32	70	22	<1	0·03	2,200	10	9	1
30.4.57	21	50	9	<1	<0·01	4	40	11	1
9.5.57	13	40	10	<1	<0·01	5	35	18	1
16.5.57	220	470	250	2·5	0·01	660	12	3	0
23.5.57	94	190	50	3·7	0·02	48	14	5	0
21.6.57	31	80	18	4·4	<0·01	340	2,130	5	1
19.7.57	112	110	18	1·2	<0·01	2,460	45	18	1
1.8.57	39	100	17	1	<0·01	3,400	60	17	6
29.8.57	270	70	18	1	0·01	4,800	50	10	1
19.9.57	100	40	6	1	0·01	12,000	40	14	1
Cows brought indoors 10.11.57									
22.11.57	—	8,100	180	1	—	300	200	160	3
6.12.57	17	5,000	7,200	375	3·75	40	200	200	16
17.12.57	16	11,200	5,500	3,500	1·06	150	1,400	1,300	100
2.1.58	6	2,300	1,010	103	0·03	17	350	85	20
16.1.58	15	160	162	19	0·01	2,420	150	125	2
28.1.58	29	80	37	—	0·01	4,880	540	60	2
12.2.58	80	66,000	16,000	180	1·56	4,800	280	160	3

The method of examining the samples of faeces was as follows. Two grammes of faeces were added to 25 ml of quarter-strength Ringer's solution in a 2 oz medical flat containing glass beads. The mixture was shaken thoroughly for 10 min, left for 1 hr, reshaken and then filtered through sterile cotton wool. Ten ml of the filtrate were added to 500 ml of sterile milk and the whole again shaken thoroughly. This faeces-infected milk was used for duplicate determinations of: (a) colony count (YMA, 3 days at 30°); (b) pasteurized milk count, i.e. milk heated to 63·5° for 35 min, cooled and plated (YMA, 3 days at 30°); (c) spore count (100° for 2 min), plated (YMA, 2 days at 37·5°); (d) spore count (100° for 15 min), plated (YMA, 2 days at 37·5°); (e) resistant spore count (105° for 15 min). The milk was dispensed in 1×50 ml, 5×10 ml, 5×1 ml and 5×0·1 ml amounts (using litmus milk as diluent), heated in a pressure cooker at 100° for 10 min, followed by 105° for 15 min. The samples were subsequently incubated at 30° for 14 days and examined for bacteriological fault.

The sample of the producer's milk was subjected to the tests (a), (b), (c) and (d) described above and the results of this work are given in Table 5. They indicate that the producer's milk normally contained relatively few non-sporing thermoduric organisms (the only exception being the milk sampled on 21.6.57). This producer was usually most conscientious about steaming his utensils.

Table 6. *Mean spore counts obtained from the* 12 *visits during indoor feeding and* 11 *visits during outdoor feeding*

Heat treatment	Indoor feeding	Outdoor feeding
	Faeces (spores/g)	
None (spores & vegetative cells)	46,200,000	86,500,000
63·5°, 35 min	12,600,000	156,000
100°, 2 min	4,400,000	49,000
100°, 15 min	502,000	1,300
105°, 15 min	677	7
	Milk (spores/ml)	
None (spores & vegetative cells)	1,160,000	2,360,000
63·5°, 35 min	377	244
100°, 2 min	256	29 (11)*
100°, 15 min	18	3 (1)*

* The bracketed figures are means obtained by disregarding the samples taken on 18.4.57, which may have included a 'carry-over' of udder contamination from indoor feeding two days previously.

These results, which are summarized in Table 6, confirm the lower spore content of bovine faeces when cattle are not housed. On the average, the bovine faeces contained about a hundred times more spores during the winter than the summer. A faecal infection of the milk in the ratio of $1:10^4$ would infect the milk with spores at approximately the same concentration as they are found in bulk supplies, and it may be significant that the amount of slime removed from clarifiers, and which contains an appreciable amount of faecal material, is also of the same order. There seems to be some evidence therefore that bovine faeces could be a primary source of spore formers in milk supplies.

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References

Burton, H., Akam, D. N., Thiel, C. C., Grinsted, E. & Clegg, L. F. L. (1953). Some investigations into the batch process of milk sterilization. *J. Soc. Dairy Tech.* **6**, 98.

Candy, M. R. & Nichols, A. A. (1956). Some bacteriological aspects of commercially sterilized milk. II. Type of spore forming bacteria isolated. *J. Dairy Res.* **23**, 329.

Franklin, J. G., Williams, D. J. & Clegg, L. F. L. (1956). A survey of the number and types of aerobic mesophilic spores in milk before and after commercial sterilization. *J. appl. Bact.* **19**, 46.

Grinsted, E. & Clegg, L. F. L. (1955). Spore forming organisms in commercial sterilized milk. *J. Dairy Res.* **22**, 178.

Ridgway, J. D. (1954). A note on the seasonal variation of the keeping quality of commercial sterilized milk. *J. appl. Bact.* **17**, 1.

Ridgway, J. D. (1955). Some recent observations on the bacteriology of sterilized milk. *J. appl. Bact.* **18**, 374.

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Cosslett, V. E. (1958). *J. appl. Bact.* **21** (1), 128–136.

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RECENT ADVANCES IN ELECTRON MICROSCOPICAL TECHNIQUES IN MICROBIOLOGY

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Contents

		page
1.	Introduction	128
2.	Instrumental	129
	(a) Resolving power	129
	(b) Illumination	129
	(c) Speed and reliability of operation	129
3.	Preparative methods for isolated organisms	130
	(a) Specimen mounting	130
	(b) Freeze-drying	130
	(c) Enzymatic and other treatments	131
	(d) Staining	131
	(e) Optical comparison	131
	(f) Replicas	132
	(g) Sectioning	132
4.	Conclusion	133
5.	Acknowledgements	133
6.	References	133

1. Introduction

A great many advances both in the electron microscope itself and in specimen techniques have been made since the previous review of the subject (Cosslett, 1952). In order to keep within reasonable length, the present review will be confined to the more important developments in experimental methods, and specific applications will be quoted only to illustrate their usefulness. A fuller account of specimen techniques has recently been published (Cosslett & Horne, 1955). Detailed references to microbiological applications will be found in Anderson (1956), and in articles contributed by various authors to the Symposium of the Society for General Microbiology on 'Bacterial Anatomy' (1956, Cambridge: The University Press), the Symposium on 'The Formation and Germination of Bacterial Spores' held by the Society for Applied Bacteriology (1957, *J. appl. Bact.* **20**, pp. 305–424) and the proceedings of the Stockholm Electron Microscopy Conference (1957, Stockholm: Almqvist & Wiksell).

2. Instrumental

The electron microscope, as commercially available, has been much improved in resolving power, reliability and speed of operation. The best instruments now show a resolution of better than 1 mμ, are in almost continuous operation and can provide photographs in rapid succession at direct magnifications up to 100,000×.

(a) *Resolving power*

Although it is not yet possible to correct spherical aberration in electron lenses, progress has been made in removing the astigmatism which is caused by lack of rotational symmetry in the lens elements. This is achieved partly by better machining and partly by incorporating correcting fields in the lens; these are variable in strength and azimuth and so can be set to compensate any residual astigmatism. These stigmators employ either electrostatic or magnetic elements and, although they are somewhat complicated in design and function, are simple to operate. So long as the electron illumination is adequate, their effect can be followed by visual observation of the image on the fluorescent screen down to a limit of 1 mμ. For very high resolution work, at present practicable only on inorganic preparations, photographic recording of a through-focal series is still needed.

The attainment of resolutions of this order demands great stability in the electrical supplies, as well as freedom from external magnetic fields and mechanical vibration. Great care has to be taken in the design of the circuits and the structure and shielding of the microscope column. In operation it is desirable to let the instrument run for some time, so as to reach thermal equilibrium, before taking photographs. In the best conditions, with subjects of high contrast, a resolution of 1·5 mμ can be obtained with biological specimens.

(b) *Illumination*

High resolution implies high working magnification, if full use is to be made of the instrument, and this in turn calls for great intensity of illumination if exposure times are to be kept to a few seconds. These needs have been met by improving the electron gun, so that the full beam output is used, and by installing a double instead of a single condenser lens. In this way greater flexibility of illuminating conditions is obtained and it becomes possible to illuminate only a very small area of the specimen, a few microns in diameter, thereby reducing heating effects. The required high final magnification is attained by using two projector lenses, of low and high power respectively, following a high power objective. The new Metropolitan-Vickers EM6 model (Plate 1, Fig. 1) has a maximum direct magnification of 100,000×, the Siemens Elmiskop (Plate 1, Fig. 2) of 160,000×, although at the latter value the illumination is so low as to require a dark-adapted eye. It is more usual to operate at 40,000–80,000×, depending on the nature of the specimen, employing a viewing telescope (×7 or ×10) for fine focusing. As normal lantern plates are used for recording the image, final photographic enlargements at 1 million or more are now commonplace.

(c) *Speed and reliability of operation*

Reliability has come mainly as a result of experience in manufacturing and of the weak points in operation. Speed has been improved by simplifying the lay-out of

controls and operating procedures, but especially by improving the facilities for photography. It is now possible to take 6 or 12 pictures at one loading of the camera, or much greater numbers if roll-film is used. While it cannot be said that the high resolution models are as simple to use as an optical microscope, there now exist models of more limited performance (3–5 mμ) such as the Philips EM 75 which are entirely straightforward and do not require the attention of a special operator.

3. Preparative Methods for Isolated Organisms

A wide variety of methods has been devised for preparing micro-organisms for electron microscopy (Drummond, 1950; Anderson, 1956). It is desirable to make the broad division between embedded and sectioned material and that simply mounted on thin membranes for direct observation, either of whole cells or of fragments produced by mechanical or enzymatic treatment. The necessity for drying specimens before they can be put into the instrument, and also the fact that some material such as spores is too thick for the electron beam to penetrate, has led to the development of a number of specialised procedures. The principles only will be mentioned here; experimental details are available in the original papers.

(a) *Specimen mounting*

The use of thin collodion or Formvar membranes for supporting the specimen has largely been superseded by the carbon film, which is practically structureless and extremely resistant to damage in the electron beam. It is prepared (Bradley, 1954, 1956) by evaporation from an incipient arc between spectroscopically pure carbon rods, in vacuo. The carbon is received on a glass slide, from which it is then floated off in water and picked up on the standard mesh disc. Alternatively it can be deposited on a disc previously covered with Formvar or nitrocellulose, which is subsequently dissolved away. By these methods films as thin as 25–50Å may be prepared. For supporting sections it is desirable to have openwork films if high resolution is the aim, and these may be obtained by evaporating carbon on to Formvar or nitrocellulose nets prepared in the manner described by Jaffe (1948) and Sjöstrand (1957).

In the case of micro-organisms, a small droplet of the suspension is usually deposited on the supporting film with a wire loop or micro-pipette. A micro-manipulation technique has been described for this purpose (Sugar, 1957). Valentine & Bradfield (1956) have devised a drainage procedure for removing cell debris or other foreign matter. A spraying technique has been devised by Backus & Williams (1950), with which droplets only a few microns in diameter are formed (Plate 1,Fig. 3). It is especially useful for controlled counts of virus particles (see also Nixon & Fisher, 1958).

(b) *Freeze-drying*

It has been found that biological structure is often best preserved if drying is carried out at a low temperature. In the original method for micro-organisms (Wyckoff, 1946) the suspension was transferred to specimen supports already at low temperature. Anderson (1954, 1956) devised a quite different method in order to avoid the passage of a phase-front through the material, with danger of artefact

formation. The suspended material is passed through alcohols and amyl acetate and finally into liquid carbon dioxide under pressure, at 25°. The temperature is then raised above the critical point of carbon dioxide (31°) and the gas allowed to escape. In this way he has obtained remarkably three-dimensional electron micrographs of human red cells and of phage-infected bacteria, which are especially impressive when seen in stereoscopic projection.

An improved version of the normal freeze-drying technique has been described by Williams (1953), in which the suspension is sprayed on to a pre-cooled copper block covered with a collodion film. After sublimation of the water content, the film is stripped off and portions mounted on mesh discs in the usual way.

(c) *Enzymatic and other treatments*

Increasing use is being made of chemical and mechanical means of breaking down organisms into their structural components, for further morphological and chemical analysis. Salton & Horne (1951*a*,*b*) have used treatment with hot water and mechanical disintegration to study the cell wall of bacteria, and Newton & Horne (1957, 1958) that of *Strigomonas oncopelti*. Peters (1956) and Peters & Stoeckenius (1956) used enzymic digestion with pepsin, papain and other agents to investigate the substructure of animal viruses. Similar methods have now been applied to bacteriophage by Brenner (1958) (Plate 1, Fig. 4); by treatment with papain and ultracentrifugation, for instance, the tail sheaths may be isolated as a pure suspension. Controlled degradation by such means promises to be a most valuable tool in studying the biochemical significance of the fine structure of micro-organisms.

(d) *Staining*

An equally important approach is to use heavy metal compounds as stains, in analogy with the optical procedures. To give appreciable enhancement of contrast in the electron image, such a reagent must have high density, preferably high atomic number, and massive attachment (preferably specific) to cell constituents (Zeitler & Bahr, 1957; Valentine, 1958). Compared with optical techniques, electron microscope staining is still in its infancy. Hall (1955) has investigated quantitatively the adsorption of osmium tetroxide and phosphotungstic acid into plant viruses, and Bahr, Bloom & Friberg (1957) that of OsO_4 into tissues. Specific attachment has been proven in very few cases, although use has been made of the well-known affinity of nucleic acids for lanthanum salts. Gibbons & Bradfield (1957) have obtained improved micrographs of chromosomes by this means (Plate 2, Fig. 5). Kellenberger & Ryter (1958) have similarly employed uranyl acetate on bacteriophage, so that they appear almost too dense (Plate 3, Fig. 6). A great deal of patient work is required before it will be possible to identify even the simplest chemical constituents of the cell by electron microscopy. Valentine (1958) has pointed out that compounds which do not contain a heavy element may be usefully used if their local absorption is sufficiently massive.

(e) *Optical comparison*

The value of comparing their results with optical micrographs is perhaps not often enough recognized by electron microscopists. It is particularly important in connection with the identification of functional units, such as nuclei or mitochondria,

for which specific optical staining methods are available. It is not difficult to work out a procedure for examining the same cells first under an immersion objective and then in the electron microscope (Brieger & Glauert, 1956), as illustrated in Plate 2, Fig. 7. In the case of embedded organisms it is possible to cut alternate thick and thin sections (Gibbons & Bradfield, 1956) so that essentially the same sample can be examined by ultraviolet spectrometry and in the electron microscope. By this means it was shown that the standard method of fixation in osmium tetroxide and embedding in methacrylate was not the cause of the lack of visibility of chromosomes in the electron micrograph (Plate 2, Fig. 8). The reason lies in the poor natural contrast of chromosomes with respect to the rest of the nucleus and their inability to take up osmic acid.

(f) *Replicas*

Replica methods for studying surface relief have been highly developed for metallurgical and other inorganic applications, but only lately have they been used on biological material. The carbon evaporation method mentioned above can be readily adapted to this end (Bradley & Williams, 1957). Its application to the surface structure of bacterial spores (Plate 2, Fig. 9) has been reported to an earlier Symposium of the Society (Franklin & Bradley, 1957). It has also been applied to a study of yeast cells and of certain algae (Harris & Bradley, 1956*a*, *b*). Replicas of a *Spirillum* have been obtained with a modification of this method (Coman & Anderson, 1955), involving shadowing with chromium, by Anderson (1956).

(g) *Sectioning*

For reasonable clarity of detail in the electron micrograph, the specimen must be thinner than about 1000Å (100 mμ). In the interests of high resolution it should be still thinner (Cosslett, 1957). The study of bulk tissue has therefore stimulated the development of improved methods of microtomy, so that sections as thin as 10 mμ can now regularly be cut. A considerable number of designs has been developed, particularly to avoid picking up the section from the knife on the return stroke. The instruments most used are those due to Porter & Blum (1953), Sjöstrand (1953), Hodge, Huxley & Spiro (1954), Haanstra (1955), Sitte (1955) and Huxley (1957). Almost all of them prefer the use of a sliver of broken glass (Latta & Hartmann, 1950) to the conventional microtome blade. Fernandez-Moran (1956) has introduced the diamond knife, which has advantages over the glass type especially in working life.

The preparative techniques are still in the phase of active development, and considerable differences of opinion exist as to the best methods for a particular material. For bulk tissue fixation in osmium tetroxide at pH 7, with phosphate buffer, is generally favoured rather than formalin, acetic acid or other organic liquid. Gibbons & Bradfield (1957) found, on the other hand, that formalin gives much better preservation of nuclear material in spermatids, even without the use of lanthanum or other salts. Kellenberger & Ryter (1958) prefer osmium tetroxide for studies of phage-infected bacteria, but insist on veronal buffer and claim that the presence of calcium ions is necessary for proper preservation of structure (Plate 3, Fig. 10).

Opinions also differ on the best embedding material to use, though almost all are

agreed on the necessity for something that sets extremely hard if very thin sections are to be cut. A mixture of butyl and methylmethacrylate monomers, in varying proportions, has been much favoured, with the addition of benzoyl peroxide to accelerate polymerisation; details are given by Sjöstrand (1956). Recently an epoxy-resin, known under the trade name of Araldite, has been found to avoid some of the minor artefacts which sometimes appear with methacrylate (Maaløe & Birch-Anderson, 1956; Glauert, Rogers & Glauert, 1956; Glauert & Glauert, 1958). It consists of two components which set rock-hard on mixing; care has to be taken to get a variety which is sufficiently liquid for reasonably rapid embedding (Plate 4, Fig. 11). A different material again, a polyvinylester known as Vinox, is favoured by Kellenberger, Schwab & Ryter (1956); again, not all batches have the right combination of properties.

The ultra-thin section technique offers the possibility of investigating the development of infective organisms *in situ* in the invaded tissue, but so far little has been published in this field. Greater attention has been paid to the study of the internal structure of bacteria, most of which are too thick to show fine detail when examined whole. A recent Symposium ('Bacterial Anatomy', 1956; Cambridge: The University Press) surveyed the progress which has been made. Bradfield (1954) has shown, for instance, that cocci have a structure in every way analogous to that of the classical cell, probably including a central nucleus (Plate 4, Fig. 12). Brieger, Cosslett & Glauert (1954) and Brieger & Glauert (1956) have studied the variation in the tubercle bacillus with time and with different growth media, and Brieger (1958) has now turned his attention to the leprosy organism.

4. Conclusion

It is a little surprising, perhaps, that so far hardly anything has been done in the field of applied bacteriology, possibly because a great deal of ground work is still needed. The detailed investigation by Kellenberger (1953), of the variation in the appearance of *Escherichia coli* from different growth media and after various treatments, marks one direction in which electron microscopy must follow the critical procedures of optical microscopy. The recent thorough study by Van Iterson (1958) of *Gallionella ferruginea*, found in connection with iron pipes, is another such indication. But by and large it must be concluded that it will be some time before electron microscopy becomes a regular routine method for the applied bacteriologist. At the moment it is roughly in the stage which optical microscopy had reached by the middle of the nineteenth century.

5. Acknowledgements

My thanks are due to all those who have supplied me with information on their recent work, and particularly to those who so willingly provided micrographs.

6. References

Anderson, T. F. (1954). Some fundamental limitations to the presentation of three-dimensional specimens for the electron microscope. *Trans. N.Y. Acad. Sci.* **16**, 242.

Anderson, T. F. (1956). In *Physical Techniques in Biological Research*, Vol. III, Chap. 5, Electron microscopy of micro-organisms. New York: Academic Press.

Backus, R. C. & Williams, R. C. (1950). The use of spraying methods and of volatile suspending media in the preparation of specimens for electron microscopy. *J. appl. Phys.* **21**, 11.

Bahr, G. F., Bloom, G. & Friberg, U. (1957). Problems of osmium fixation. *Proc. 1st Europ. Reg. Conf. Electron Microscopy, Stockholm*, p. 106. Stockholm: Almqvist & Wiksell.

Bradfield, J. R. G. (1954). Electron microscopic observations on bacterial nuclei. *Nature, Lond.*, **173**, 184.

Bradley, D. E. (1954). Evaporated carbon films for use in electron microscopy. *Brit. J. appl. Phys.* **5**, 65.

Bradley, D. E. (1956). Uses of carbon replicas in electron microscopy. *J. appl. Phys.* **27**, 1399.

Bradley, D. E. & Williams, D. J. (1957). An electron microscope study of the spores of some species of the genus *Bacillus* using carbon replicas. *J. gen. Microbiol.* **17**, 75.

Brieger, E. M. (1958). The electron microscopy of *Mycobacterium leprae*. In *Leprosy in Theory and Practice*, edited by R. G. Cochrane. Bristol: John Wright & Son.

Brieger, E. M., Cosslett, V. E. & Glauert, A. M. (1954). Reproductive changes in avian tubercle bacilli studied with the electron microscope. *J. gen. Microbiol.* **10**, 294.

Brieger, E. M. & Glauert, A. M. (1956). The internal structures of mycobacteria studied by comparative microscope methods. *Int. Conf. Electron Microscopy, London*, p. 330. London; Royal Microscopical Society.

Brenner, S. (1958). Unpublished work.

Coman, D. R. & Anderson, T. F. (1955). A structural difference between the surfaces of normal and of carcinomatous epidermal cells. *Cancer Res.* **15**, 541.

Cosslett, V. E. (1952). The application of electron microscopy in cytology. *Chem. & Ind.* **30**, 29.

Cosslett, V. E. (1957). How thin should a section be? *J. biophys. biochem. Cytol.* **3**, 815.

Cosslett, V. E. & Horne, R. W. (1955). Recent developments in specimen preparation techniques for electron microscopy. *Vacuum* **5**, 109 (published November 1957).

Drummond, D. G. (1950). The practice of electron microscopy. *J. R. micr. Soc.* **70**, Part 1.

Fernandez-Moran, H. (1956). Applications of a diamond knife for ultrathin sectioning to a study of the fine structure of biological tissues and metals. *J. biophys. biochem. Cytol.* **2** (Suppl.), 29.

Franklin, J. G. & Bradley, D. E. (1957). A further study of the spores of species of the genus *Bacillus* in the electron microscope using carbon replicas, and some preliminary observations on *Clostridium welchii*. *J. appl. Bact.* **20**, 467.

Gibbons, I. R. & Bradfield, J. R. G. (1956). The fixation of cell nuclei by osmium tetroxide. *Biochem. biophys. Acta* **22**, 506.

Gibbons, I. R. & Bradfield, J. R. G. (1957). The fine structure of nuclei during sperm maturation in the locust. *J. biophys. biochem. Cytol.* **3**, 133.

Glauert, A. M. & Glauert, R. H. (1958). Araldite as an embedding medium for electron microscopy. *J. biophys. biochem. Cytol.* **4**, 191.

Glauert, A. M., Rogers, G. E. & Glauert, R. H. (1956). A new embedding medium for electron microscopy. *Nature, Lond.*, **178**, 803.

Haanstra, H. B. (1955). A simple ultramicrotome. *Philips tech. Rev.* **17**, 178.

Hall, C. E. (1955). Electron densitometry of stained virus particles. *J. biophys. biochem. Cytol.* **1**, 1.

Harris, K. & Bradley, D. E. (1956*a*). Electron microscopy of synura scales. *Discovery* **17**, 329.

Harris, K. & Bradley, D. E. (1956*b*). An examination of the scales and bristles of *Mallomonas* in the electron microscope using carbon replicas. *J. R. micr. Soc.* **76**, 37.

Hodge, A. J., Huxley, H. E. & Spiro, D. (1954). Electron microscope studies on ultra-thin sections of muscle. *J. exp. Med.* **99**, 201.

Huxley, A. F. (1957). An ultramicrotome. *J. Physiol.* **137**, 73P.

van Iterson, W. (1958). *Gallionella ferruginea* Ehrenberg in a different light. Thesis, Amsterdam.

Jaffe, M. S. (1948). Auxiliary supporting nets for fragile electron microscope specimens. *J. appl. Phys.* **19**, 1191.

Kellenberger, E. (1953). Les formes charactéristiques des nucléoides de *E. coli* et leurs transformations dues a l'action d'agents mutagènes-inducteurs et de bactériophages. *Symposium on Bacterial Cytology, Int. Congr. Microbiol., Rome*, p. 45. Rome: Fond. Em. Paterno.

Kellenberger, E., Schwab, W. & Ryter, A. (1956). L'utilisation d'un copolymère du groupe des polyesters comme matériel d'inclusion en ultramicrotomie. *Experientia* **12**, 521.

Kellenberger, E. & Ryter, A. (1958). Cell wall and cytoplasmic membrane of *E. coli*. *J. biophys. biochem. Cytol.* (in publication).

Latta, H. & Hartmann, J. F. (1950). Use of a glass edge in thin sectioning for electron microscopy. *Proc. Soc. exp. Biol., N.Y.*, **74**, 436.

Maaløe, O. & Birch-Anderson, A. (1956). On the organisation of the 'nuclear material' in *Salmonella typhimurium*. *Symp. Soc. gen. Microbiol.* no 6. *Bacterial Anatomy*, p. 261. Cambridge: The University Press.

Newton, B. A. & Horne, R. W. (1957). Intracellular structures in *Strigomonas oncopelti*. I. Cytoplasmic structures containing ribonucleoprotein. *Exp. Cell. Res.* **13**, 563.

Newton, B. A. & Horne, R. W. (1958). Intracellular structures in *Strigomonas oncopelti*. II. Fine structure of the kinetoplast-blepharoplast complex. *Exp. Cell. Res.* (in the press).

Nixon, H. L. & Fisher, H. L. (1958). An improved spray droplet technique for quantitative electron microscopy. *Brit. J. appl. Phys.* **9**, 68.

Peters, D. (1956). Morphology of resting vaccinia virus. *Nature, Lond.*, **178**, 1453.

Peters, D. & Stoeckenius, W. (1956). Innenstrukturen des Vaccine-Virus. *Int. Conf. Electron Microscopy, London*, p. 237. London: Royal Microscopical Society.

Porter, K. R. & Blum, J. (1953). A study in microtomy for electron microscopy. *Anat. Rec.* **117**, 685.

Salton, M. R. J. & Horne, R. W. (1951*a*). Studies of the bacterial cell wall. I. Electron microscopical observations on heated bacteria. *Biochim. biophys. Acta* **7**, 19.

Salton, M. R. J. & Horne, R. W. (1951*b*). Studies of the bacterial cell wall. II. Methods of preparation and some properties of cell walls. *Biochim. biophys. Acta* **7**, 117.

Sitte, H. (1955). Ein einfaches Ultramikrotom für hochauflösende elektronenmikroskopische Untersuchungen. *Mikroskopie* **10**, 365.

Sjöstrand, F. S. (1953). A new microtome for ultrathin sectioning for high resolution electron microscopy. *Experientia* **9**, 114.

Sjöstrand, F. S. (1956). In *Physical Techniques in Biological Research*, Vol. III, Chap. 6, Electron microscopy of cells and tissues. New York: Academic Press.

Sjöstrand, F. S. (1957). An improved method to prepare Formvar nets for mounting thin sections for electron microscopy. *Proc. 1st Europ. Reg. Conf. Electron Microscopy, Stockholm*, p. 120. Stockholm: Almqvist & Wiksell.

Sugar, I. (1957). A micro-manipulation method for the preparation of calibrated microdroplets over the range 10^{-6}–10^{-10} ml in electron microscopy. *Proc. 1st Europ. Reg. Conf. Electron Microscopy, Stockholm*. p. 127. Stockholm: Almqvist & Wiksell.

Valentine, R. C. (1958). Contrast in the electron microscope image. *Nature, Lond.*, **181**, 832.

Valentine, R. C. & Bradfield, J. R. G. (1956). A simple method for preparing biological specimens in suspension for electron microscopy. *Int. Conf. Electron Microscopy, London*, p. 148. London: Royal Microscopical Society.

Williams, R. C. (1953). A method of freeze-drying for electron microscopy. *Exp. Cell. Res.* **4**, 188.

Wyckoff, R. W. G. (1946). Frozen-dried preparations for the electron microscope. *Science* **104**, 36.

Zeitler, E. & Bahr, G. F. (1957). Contributions to the quantitative interpretation of electron microscope pictures. *Exp. Cell. Res.* **12**, 44.

Explanation of Plates

Plate 1

Fig. 1. Metropolitan-Vickers electron microscope, model EM6.

Fig. 2. Siemens-Halske electron microscope, model Elmiskop I.

Fig. 3. Typical spray droplet, containing tobacco mosaic virus rods and polystyrene latex spheres. Shadowed with palladium; ×12,000. (Reproduced by permission of the Editor, *British Journal of Applied Physics*, from Nixon, H. L. & Fisher, H. L., *Brit. J. appl. Phys.* **9**, 68, 1958; Fig. 2).

Fig. 4. T2 bacteriophage, isolated from *E. coli* and treated with deoxyribonuclease; the heads are empty ghosts and some of the tail structure is visible. Shadowed with chromium; ×31,000. (Brenner, S. & Horne, R. W., Cavendish Laboratory, Cambridge; hitherto unpublished).

PLATE 2

Fig. 5. Longitudinal section of metaphase chromosomes from locust testis, fixed with formaldehyde and stained with lanthanum nitrate; ×13,500. (Gibbons, I. R. & Bradfield, J. R. G., Cavendish Laboratory, Cambridge; hitherto unpublished).

Fig. 7. Comparison of optical (upper, ×3,500) and electron (lower, ×10,000) micrographs of the same filaments of *Mycobacterium phlei*. Optical, stained with May-Grünwald and Giemsa. The electron-dense bodies correspond to the red-staining metachromatic granules. (Reproduced by permission of the Royal Microscopical Society, from Brieger, E. M. & Glauert, Audrey M., *Int. Conf. Electron Microscopy, London*, p. 330; Fig. 1a, b).

Fig. 8. Thin section of osmium fixed nucleus in locust testis: (a) electron micrograph (×9,000) compared with (b) ultraviolet micrograph (×3,000) of the same nucleus in an adjacent thicker section. (Reproduced by permission of the Elsevier Publishing Company, from Gibbons, I. R. & Bradfield, J. R. G., *Biochim. biophys. Acta.* **22**, 506, 1956; Fig. 3).

Fig. 9. Carbon replica of spores of *Bacillus macerans*, shadowed with gold-palladium; ×3,500. (Franklin, J. G. & Bradley, D. E., *J. appl. Bact.* **20**, 467, 1957, Fig. 1b.)

PLATE 3

Fig. 6. Section of *E. coli* infected with T2 bacteriophage, fixed 8 hr in osmium tetroxide at pH 6, with veronal buffer and calcium chloride, and washed in uranyl acetate; ×27,500. (Kellenberger, E. & Ryter, A., Laboratoire de Biophysique, Université de Genève; hitherto unpublished).

Fig. 10. *E. coli*, fixed 16 hr in osmium tetroxide at pH 6, with veronal buffer and uranyl acetate. The nucleoplasm is very fine, the granulation of the cytoplasm hardly visible and the cell wall very marked; ×27,500. (Kellenberger, E. & Ryter, A., Laboratoire de Biophysique, Université de Genève; hitherto unpublished).

PLATE 4

Fig. 11. Thin sections of *Streptomyces coelicolor*, fixed in osmic acid and embedded in (a) *n*-butyl methacrylate (×39,000) and (b) Araldite, an epoxy resin (×40,500). The vacuole surrounding the central dense structure is far less marked in Araldite and is partially an artefact of methacrylate embedding. The outer cell wall is also better preserved in Araldite and remains more closely apposed to the cell. (Glauert, Audrey M., Strangeways Laboratory, Cambridge, and Hopwood, D. A., Botany School, University of Cambridge; hitherto unpublished).

Fig. 12. (a) Section of *Staphylococcus aureus*, showing central nucleus containing fibrous material and surrounded by granular cytoplasm (×70,000). (b) Similar section of cocci stained with modified Feulgen, the central cluster of silver granules indicating presence of deoxyribonucleic acid (×85,000.) (Reproduced by permission of the Editors of *Nature*, from Bradfield, J. R. G., *Nature, Lond.*, **173**, 184, 1954; Figs. 2 and 4).

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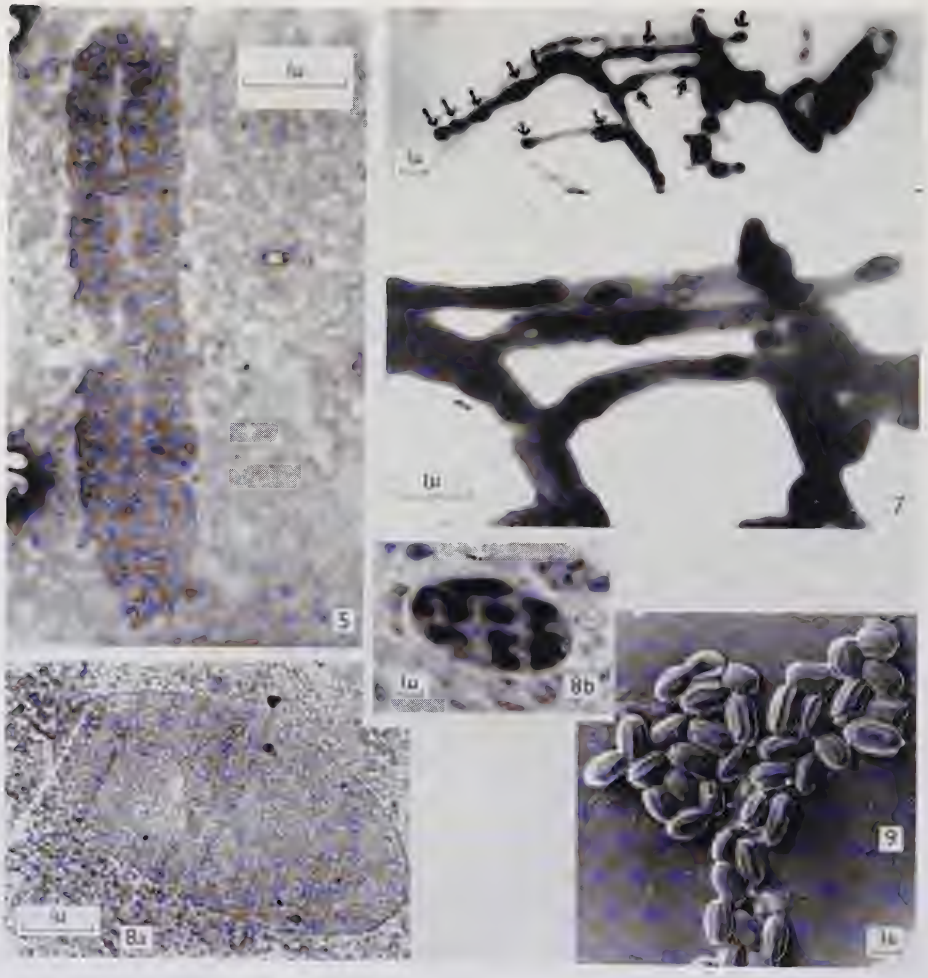
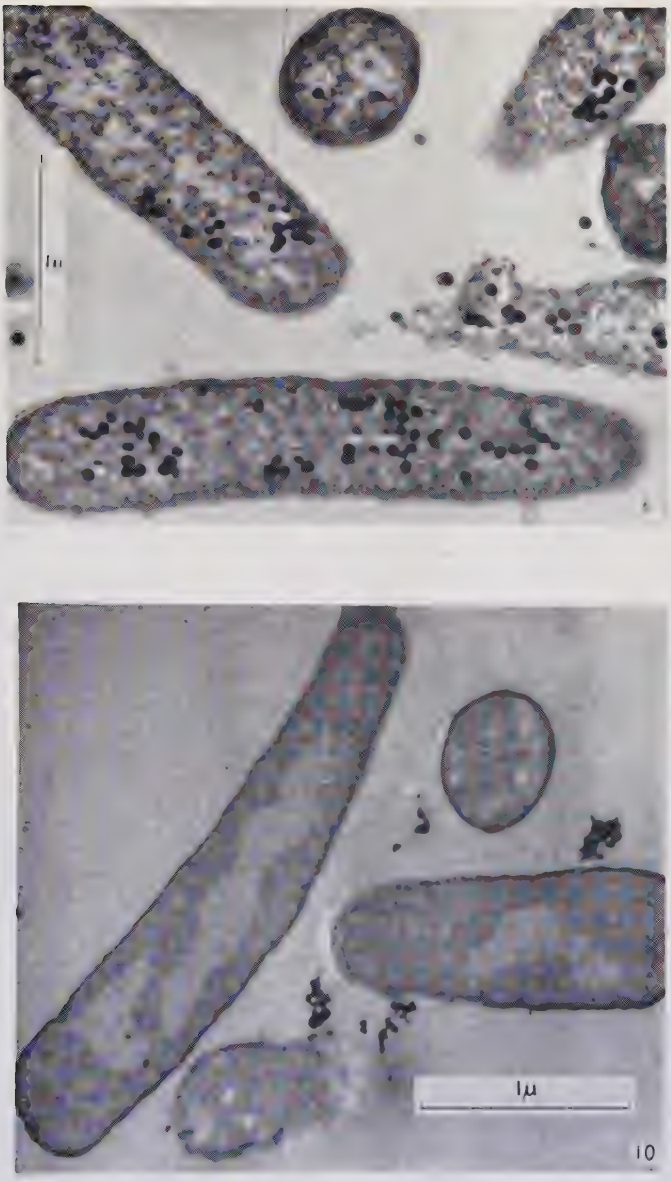
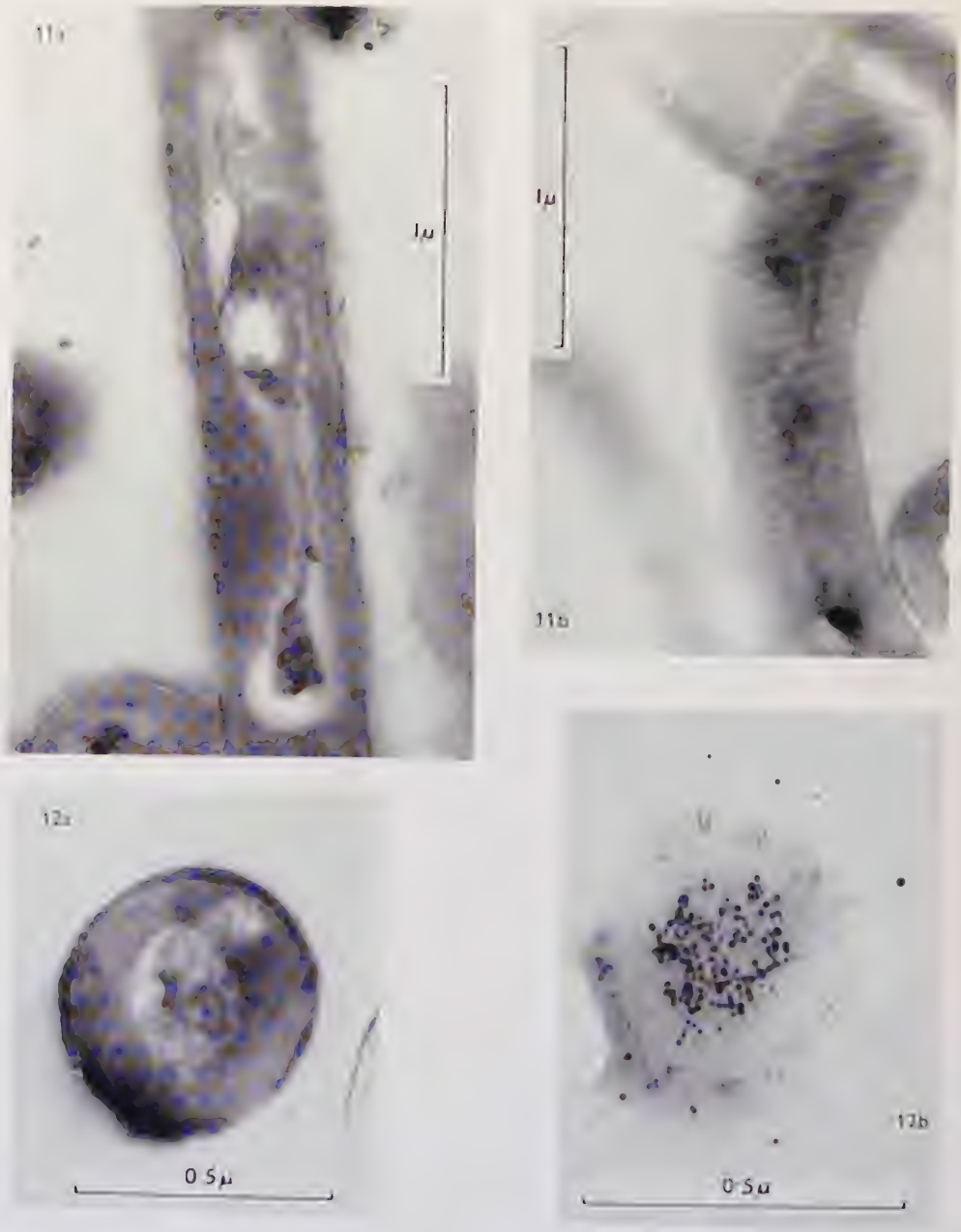
V. E. Cosslett — Recent Advances in Electron Microscopical Techniques in Microbiology Plate I



V. E. Cosslett — Recent Advances in Electron Microscopical Techniques in Microbiology. Plate 2



V. E. Cosslett — Recent Advances in Electron Microscopical Techniques in Microbiology. Plate 3



V. E. Cosslett — Recent Advances in Electron Microscopical Techniques in Microbiology. Plate 4

Smiles, J. (1958). *J. appl. Bact.* **21** (1), 137–142.

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ULTRAVIOLET MICROSCOPY

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Contents

		PAGE
1.	Introduction	137
2.	The earliest developments	137
3.	The application of dark-ground illumination	139
4.	Post-1945 developments and the use of phase-contrast	140
5.	The introduction of reflecting objectives	140
6.	'Ultraviolet staining'	141
7.	References	142

1. Introduction

The practical realization of the use of ultraviolet as a means of examining the microstructures of biological objects was made possible when von Rohr, in the spring of 1902, discovered that spherically corrected and coma-free monochromatic lenses of high numerical aperture could be constructed of one material only (see von Rohr, 1904). Herschkowitsch had already produced, in an electric furnace, fused quartz of high optical quality and in sufficiently large pieces to be used for optical purposes. He published the details in 1903. Within a short time of his discovery, von Rohr designed the well-known series of monochromats consisting of fused quartz components for use with the wavelength 275 mμ emitted by the cadmium spark. The numerical aperture of the highest power was 1·25. The resolving powers of these objectives were double the value obtaining in the case of objectives of identical numerical apertures when working with daylight.

2. The Earliest Developments

Köhler, who designed the apparatus with which these lenses could be used, has described in detail the attempts which had been made to increase microscopical resolution by using ultraviolet radiations with apochromatic objectives (1904*a*,*b*). Czapski (1891) examined the possibility of using ultraviolet light and, after examining the transmission of the available optical glasses, concluded that a wavelength of 350 mμ could be used. He also stipulated that the ultraviolet image should be coincident with the visual image formed by the lens to enable the user to focus the object in the usual way. Eight years later Köhler (1899) produced a monochromator with an incandescent source by means of which objects could be

illuminated with monochromatic light of any desired wavelength. He replaced the source with a condensed magnesium spark and tested the performance of an apochromatic objective of N.A. 1·40 when the wavelength 383 mμ was used by comparing the results with those obtained with the wavelength 448 mμ. He also used the wavelength 361 mμ emitted by the cadmium spark but concluded that the gain in resolution did not justify the labour and cost of producing a system corrected for this region of the spectrum.

Köhler (1904*a*) then turned his attention to the group of intense lines of the magnesium spark at 280 mμ. With the co-operation of von Rohr, who designed a quartz fluorspar objective of low numerical aperture, Köhler demonstrated that biological objects were in the main sufficiently transparent to these wavelengths to warrant the developments which took place later. Focusing was carried out by means of a fluorescent ocular. When a sharp visual image was obtained on the fluorescent screen this ocular was replaced by one made of quartz which projected the ultraviolet image on to the photographic plate of a camera mounted above the eyepiece. Because of the opacity of optical glasses to this region of the spectrum all optical components of the illuminating system were made of quartz.

As soon as von Rohr discovered that corrected lenses could be made of one substance only, he designed a series of three lenses for use with the 280 mμ group of lines; but when the highest power was tested it was found that the results were not as good as had been expected of it, although careful checks revealed that the fault did not arise from inaccuracies in the computation of the lenses or from technical imperfections. It had been assumed that the group, which extended over a wave band of only 12 mμ, could be treated as a single line. Calculations showed, however, that these lines were sufficiently separated to introduce enough chromatic aberration to impair the image. Experiments, however, revealed that the cadmium line 275 mμ was homogeneous and of sufficient intensity to justify the design and manufacture of the series of lenses which have since been used with marked success.

In the meantime Köhler had developed a better method of focusing the object than the one he had used with the wavelength 283 mμ. Each quartz ocular was designed to project an aberration-free image on to a photographic plate situated 30 cm from it. To focus the image in the plane of the plate Köhler introduced over the quartz eyepiece a combination of quartz lenses which brought the converging rays to a focus on a fluorescent plate mounted close to the combination. The image was viewed by means of a glass magnifier. Köhler admitted, however, that the method was not altogether satisfactory because the fluorescent image tended to be diffuse. It is probable that this, and other difficulties later experienced by Barnard, were responsible for the general lack of interest in ultraviolet microscopy. Nevertheless he succeeded in demonstrating the high image-forming qualities of von Rohr's objectives and in showing that certain organellae in biological material exhibited strong absorption in the wavelength 275 mμ.

About 1914 Barnard, in this country, purchased the apparatus as originally designed by Köhler. He experimented with this for about ten years in an effort to study the morphology of living micro-organisms. He too had to admit that the exact focusing of the ultraviolet on the photographic plate was a matter of chance.

He also found that the method of adjusting and centering the illuminating system, which had been described in great detail by Köhler (1904*a*), was by no means easy. In 1923 his attention was drawn to the fact that the focal depth of the highest power objective was only of the order of 0·25 μ and it was suggested that the fine adjustment of the Zeiss microscope might not be sufficiently accurate or sensitive. Barnard at once realised that for ultraviolet microscopy to be of real practical value the difficulties which he had encountered could be overcome only by developments in microscope design and by developing techniques by which the object could be selected and focused by direct visual methods.

3. The Application of Dark-Ground Illumination

In 1926 the Beck-Barnard microscope was produced. The fine adjustment was designed to move the objective through intervals of 0·1 μ. The microscope was mounted in a horizontal position on a rigid casting which carried the Köhler illuminating system and a visual one for use in the direct examination and focusing of the object. A duplex condenser consisting of a quartz condenser mounted centrally in a bispherical dark-ground illuminator was constructed. The two systems were parfocal. A special objective changer was designed to carry the visual and ultraviolet objectives. All the objectives, each mounted permanently on its own changer, were accurately centred to one another. They were also nearly parfocal, the errors being determined and allowed for by adjustment of the fine focusing mechanism. In practice the dark-ground illuminator was focused visually on the object and required no further adjustment when changing over to ultraviolet. Using a high power visual objective a sharp dark-ground image of the object was obtained. Without moving the coarse or fine adjustment the objective was removed and replaced by a quartz one. The focus was adjusted by means of the fine focusing mechanism to compensate for the error in parfocality. A sharp ultraviolet image of the object was in this way projected on the photographic plate when the visual eyepiece was replaced by a quartz one. The full description of this equipment was delayed for ten years (see Barnard & Welch, 1936*a*).

Meanwhile Barnard had developed a simpler form of microscope. The spark source was replaced by a low pressure mercury discharge lamp. About 90% of the radiation emitted by this lamp has a wavelength of 253 mμ (Barnard & Welch, 1936*b*). The objective used with this wavelength was one designed by von Rohr for use with the cadmium line 275 mμ. Lavin (1943) used an even simpler apparatus. He dispensed with the Köhler method by using a mercury discharge lamp and a fluid filter to isolate the wavelength 253 mμ. He claimed that after dark adaptation of the eye it was possible to focus the ultraviolet image directly on a fluorescent willemite screen placed in the plane occupied by the photographic plate.

Barnard successfully applied the method of dark-ground illumination to ultraviolet microscopy. Two types of illuminator were made by the firm of R. & J. Beck. The first constructed was of the bispherical type consisting of polished magnalium surfaces. The exposures required with this system were excessively long. The second, designed by Smiles (1933), consisted of a quartz cone and a magnalium aspherical reflector. With this system the exposures were reduced to within

reasonable limits, enabling Barnard to obtain beautiful pictures of micro-organisms and the larger viruses.

4. Post-1945 Developments and the Use of Phase-Contrast

When the Second World War ended ultraviolet equipment was no longer available. A few workers in this country were, however, anxious to apply the method to problems in which they were interested and as a result the firm of Cooke, Troughton & Simms undertook the task of designing and manufacturing the apparatus. The microscope was built along the lines of the Beck-Barnard instrument, the main differences being in the design of the fine adjustment mechanism and of the objective changer, and in the arrangement of the ultraviolet and visual illuminating equipment (Taylor, 1953). Before the war the firm of Carl Zeiss had designed a sparking apparatus in which the electrodes were discs of cadmium. They were separated by a 3 mm gap and when rapidly rotated a high tension current generated a very intense condensed spark. This equipment was too cumbersome to be used with the Beck instrument. A much smaller outfit was designed and built at the National Institute for Medical Research and mounted on the base of the Beck microscope. The cadmium discs were of 3 in. diameter and were driven at 5,000 rev/min by 24-volt motors. Cooke, Troughton & Simms improved on this by using compressed air to drive the electrodes, thus reducing the risk of vibrations being transmitted to the microscope.

Ludford, Smiles & Welch (1948), when studying living malignant cells by ultraviolet microscopy, found that the chromosomes of dividing cells could not be revealed by visual dark-ground illumination and suggested that a phase-contrast method of searching and focusing the object should be adopted. Later Cooke, Troughton & Simms produced a quartz condenser fitted with a removable annulus which matched the absorbing ring in their high power visual phase-contrast objective. This method has proved to be much superior to the dark-ground method. Following a suggestion that the phase-contrast method of microscopy might be developed to use ultraviolet light instead of visual, a Zernike disc was introduced into the high power objective for use with the wavelength 275 mμ. The absorbing disc was dimensioned to match the ultraviolet image of the annulus used for visual phase-contrast. Taylor (1950), and Smiles & Dobson (1955), have used this ultraviolet phase-contrast system together with direct ultraviolet microscopy to study the fate of nucleotides in micro-organisms present in the rumen and abomasum of the sheep. Although no mention was made of the fact, it was possible to follow the cycle of morphological changes during cell division of the contents of a large streptococcus from the rumen.

5. The Introduction of Reflecting Objectives

There can be no doubt that Caspersson's pioneer work in developing the ultraviolet microspectrophotometer by which he demonstrated the existence and distribution of nucleotides in cells and tissues drew attention to the real need for achromatic lenses to cover the wavelength range of the absorption of nucleotides and proteins. Because of the difficulty in designing achromatized refracting systems, designers turned their attention to the production of reflecting objectives. These offered a

better chance of success since they are completely achromatic and can be used over a wide range of wavelengths with uncovered objects. The focusing can be carried out in any region of the visual spectrum and the system will be in focus for wavelengths in the invisible spectrum. When cover slips are used axial chromatic aberration is introduced but the errors involved can be calculated and allowed for by adjustment of the fine focusing mechanism of the microscope. Burch (1947) designed a system consisting of an aspherical and a spherical surface having an aperture of 0·65. By adding a spherical front lens he produced an immersion system of 0·95 N.A. With this the axial achromatism is maintained but chromatic aberration becomes evident in the outer part of the field. A few objectives of N.A. 0·65 were made by Burch but owing to the high degree of skill required to produce the aspherical surface it is extremely doubtful if they will ever become generally available. Similar systems have been designed using spherical surfaces, the apertures being of the same order as the Burch objective. In certain respects they are inferior to the Burch system. The substage condensers used with these objectives are similar in design. These reflecting systems are being used in ultraviolet microspectrophotometers in the U.S.A.

An important advance has been made by Grey (1950*a*,*b*, 1951), who has designed systems which combine cemented refracting components with spherical reflecting surfaces. A series of these objectives, which are spherically and chromatically corrected for a spectral range from 200 mμ to 600 mμ, are being produced in the U.S.A. The highest power objective has a numerical aperture of 1·0.

Some years before the Second World War, Bausch & Lomb produced a lens of the type suggested by Czapski (1891). This was chromatically corrected for the wavelengths 546 and 365 mμ of the mercury arc and has a numerical aperture of 1·30. The only other refracting achromats which are available are two which have recently been put on the market by Cooke, Troughton & Simms. This firm has overcome the difficulty of achromatizing high power refracting systems for considerable ranges of the ultraviolet spectrum. One of these lenses has an aperture of 1·25 and a wavelength range of 250 to 300 mμ, the other an aperture of 1·3 and a range of 300 to 400 mμ.

6. 'Ultraviolet Staining'

Köhler (1904*a*,*b*) suggested that it might be possible to extend the use of ultraviolet microscopy to the study of biological materials treated with stains. He also suggested that colourless substances which absorb in the ultraviolet might be used. Blout (1953) has also expressed the view that ultraviolet 'staining' should be used to increase the contrast in ultraviolet photomicrographs. He suggested the use of organic compounds to which the membranes of cells are permeable. Presumably he was referring to vital stains. Up to the present it does not appear that any serious attempt has been made to exploit this method. We have recently examined the possibilities by treating living cells with the fluorochrome acridine orange and photographing them at a wavelength outside the absorption band of nucleotides, using the Cooke, Troughton & Simms achromat (N.A. 1·30). The wavelength selected was the 315 mμ line of the cadmium spark. The untreated cells were highly

transparent to this wavelength but when treated with the dye the basophilic organellae were revealed with great contrast. The success of these trials suggests a number of possible uses to which 'vital ultraviolet staining' might be put. Those which are selectively taken up by DNA-containing structures only, e.g. certain acridines, might be used to enhance contrast where the DNA concentration is low when using wavelengths in the region of 260 mμ. When photographing cells with wavelengths near 260 mμ it often happens that the nuclear structures are masked by the RNA in the cytoplasm and cytoplasmic organellae. By using the nuclear 'stains' and photographing with a wavelength outside the absorption band of RNA, this difficulty would be overcome. It is possible that the use of 'vital stains' may prove useful in the study of micro-organisms, especially Gram-positive organisms which exhibit a strong general absorption at 260 mμ. Gram-negative organisms usually show weak absorption at this wavelength. By a suitable choice of 'stain' and wavelength it may be possible to add to our knowledge of the morphology of these organisms.

7. References

Barnard, J. E. & Welch, F. V. (1936*a*). *Practical Microscopy*, 3rd ed. London: Arnold & Son.

Barnard, J. E. & Welch, F. V. (1936*b*). Microscopy with ultra-violet light. A simplification of method. *J.R. micr. Soc.* **56**, 365.

Blout, E. R. (1953). *Advances in Biological and Medical Physics.* New York: Academic Press Inc.

Czapski, S. (1891). Die voraussichlichen Grenzen der Leistungsfähigkeit des Mikroskops. *Z. wiss. Mikr.* **8**, 145.

Burch, E. R. (1947). Reflecting microscopes. *Proc. phys. Soc. Lond.* **59**, 41.

Grey, D. S. (1950*a*). A new series of microscope objectives. II. Preliminary investigations of catadioptric Schwarzschild systems. *J. opt. Soc. Amer.* **39**, 723.

Grey, D. S. (1950*b*). A new series of ultra-violet objectives of intermediate numerical aperture. *J. opt. Soc. Amer.* **40**, 283.

Grey, D. S. (1951). Computed aberrations of spherical Schwarzschild reflecting microscope objectives. *J. opt. Soc. Amer.* **41**, 183.

Herschkowitsch, M. (1903). Über die Umwandlung des Bergkristalls in den amorphen Zustand. *Z. phys. Chem.* **46**, 408.

Kohler, A. (1899). Beleuchtungsapparat für gleichmässige Beleuchtung mikroskopischer Objecte mit beliebigem, einfarbigem Licht. *Z. wiss. Mikr.* **16**, 1.

Kohler, A. (1904*a*). Mikrophotographische Untersuchungen mit ultraviolettem Licht. *Z. wiss. Mikr.* **21**, 129.

Kohler, A. (1904*b*). Mikrophotographische Untersuchungen mit ultraviolet Licht. *Z. wiss. Mikr.* **21**, 273.

Lavin, G. I. (1943). Simplified ultra-violet microscopy. *Rev. sci. Instrum.* **14**, 375.

Ludford, R. J., Smiles, J. & Welch, F. V. (1948). The study of living malignant cells by phase-contrast and ultra-violet microscopy. *J.R. micr. Soc.* **68**, 1.

Smiles, J. (1933). Dark-ground illumination in ultra-violet microscopy. *J.R. micr. Soc.* **53**, 203.

Smiles, J. & Dobson, M. J. (1955). Direct ultra-violet and ultra-violet phase-contrast micrography of bacteria in the stomachs of the sheep. *J.R. mic. Soc.* **75**, 244.

Taylor, E. W. (1950). The application of phase-contrast to the ultra-violet microscope. *Proc. roy. Soc.* B. **137**, 332.

Taylor, E. W. (1953). The ultra-violet microscope. *J. opt. Soc. Amer.* **43**, 299.

Von Rohr, M. (1904). *Die Bilderzeugung in Optischen Instrumenten vom Standpunkte der Geometrischen Optik.* Berlin: J. Springer.

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Hughes, W. H. (1958). *J. appl. Bact.* **21** (1), 143–145.

(Symposium on New Techniques in Microscopy: Paper III)

THE ADVANTAGES AND USES OF PHASE CONTRAST MICROSCOPY

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It is a full ten years since the phase contrast microscopes were available in this country. It should now be possible to assess their value and the advantages that they have over the traditional equipment for light microscopy.

The examination of living organisms

The first claim made is that there is no longer any need to stain organisms for examination. This, on the face of it, is no great advantage since simple staining is quick and easy and saving a few minutes would certainly not justify the expensive equipment. There are other considerations, however. The first is that in preparing organisms by drying on a slide there is a considerable loss of volume. This may amount to as much as one-third of the diameter and up to nine-tenths of the thickness. Any interest which we may have in the internal structures of micro-organisms will be frustrated by this compression of the intracellular content. Not only so, but the plating of a layer of dye on the outside of the organism while revealing its outline will obscure any fine features. The question of resolution in microscopy has here to be considered. Although organisms under phase contrast appear larger than they would do by the traditional methods of examination it must be pointed out that dark ground microscopy still reveals structures which are invisible in any other way. Nevertheless, a comparison of the resolution of an object by the two methods reveals that the structures seen under dark ground are not better resolved than under phase contrast examination. In fact, in my hands, the latter method gives slightly better resolution than the former. It is interesting to note that with a phase contrast microscope and a simple two lens Abbé substage condenser of the type commercially available, the maximum resolution of the objective is obtained and no improvement can be hoped for by adapting fully corrected apochromats with a numerical aperture of 1·4 for this work. The reason for this apparently anomalous finding is that the phase contrast method uses a much greater proportion of the refracted light than does ordinary microscopy: the funneling of the direct rays which limits the numerical aperture no longer applies and the whole of the field seen when the back lens of the one-twelfth objective is examined is found to be fully illuminated.

The recording of changes in cultures by time-lapse cinemicrography

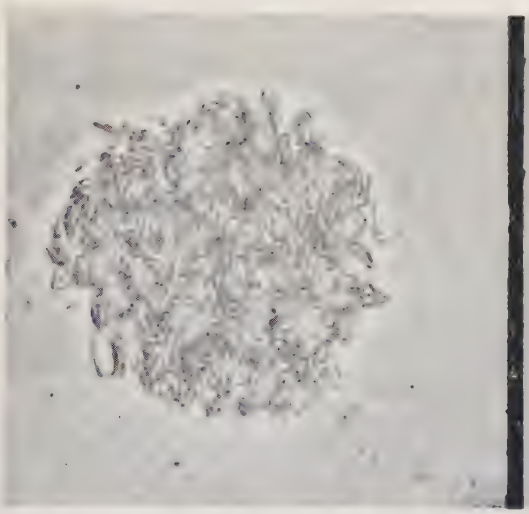
In addition to the above two factors—the visibility of organisms without staining and the full resolution of the light microscope—there is another advantage made available by phase contrast and that is its combination with the cinecamera to give time-lapse cinemicrography or indeed permanent records at ordinary speed. As long ago as the early 1930s cinemicrography was in use to record the changes taking place in cultures of micro-organisms. The records were used mainly to illustrate papers and comparison was made between single frames taken at intervals. Few, if any, films were made available for teaching purposes by this method and the technical difficulties were considerable, since the only way of making organisms visible was by racking down the substage, thereby losing a great deal of the available light. Results, however, were sufficiently good to make one wonder why the method was so little used in research at that time. It may well have been due to the relative slowness of the film emulsions then available. Today the phase contrast equipment is the tool of choice in this work and the high speed emulsions on 16 mm film make it possible to monitor the development of organisms for periods of 48 hr or more, and to compress the phases of growth into reasonable sequences lasting up to half a minute.

Description of the apparatus

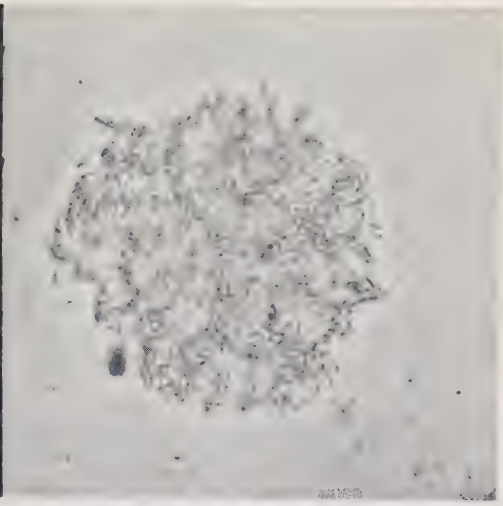
The essentials of the apparatus, which can in general be constructed in the laboratory, are first a large slab of concrete, or other stone, supported on rubber. It is essential that vibration should be prevented from reaching the microscope. The cinecamera should be supported independently of the optical system. No lens, of course, is needed, the objective of the microscope serving this purpose. Vibration from the cinecamera must not be conveyed to the microscope, but the stand on which the camera is supported need not itself be protected in any way from the room vibrations. There is only one other item of equipment which is essential and that is an insulating box arranged as an incubator and thermostatically controlled, around the microscope. Without this, expansion and contraction of the brass of the stand will continually affect the focus.

In the apparatus designed and made by Dr. P. N. Cardew, the eyepiece is a Baker $\times 1\frac{1}{2}$, which overcomes in part the tendency to spurious magnification inherent in the method. With our camera there is a factor of $\times 3$ represented by the projection from eyepiece to camera. It is important to remember this when judging the results on film; in most of our shots the primary magnification is much lower than might be expected from the projected sequences; it is seldom necessary to use any higher power objective than the 2/3 in.

Not only has it been possible to make permanent records of phenomena which are not easy to organize for demonstration purposes, but the time-lapse technique, compressing the observations of hours or days into half a minute, has the supreme advantage that it overcomes fatigue, so that things that could otherwise never be certainly recorded by simple observation become available for repeated study.



(*a*)



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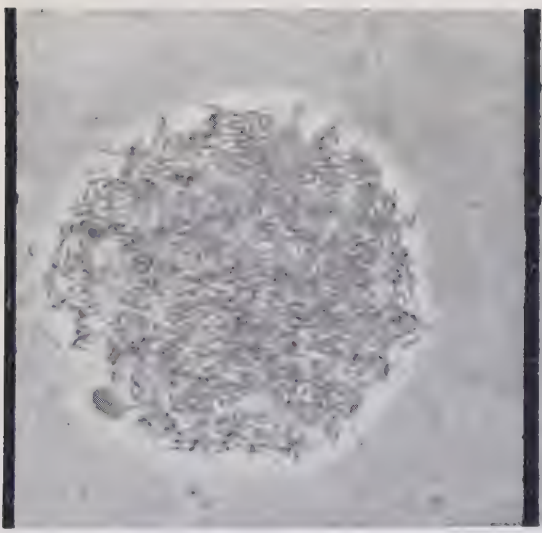

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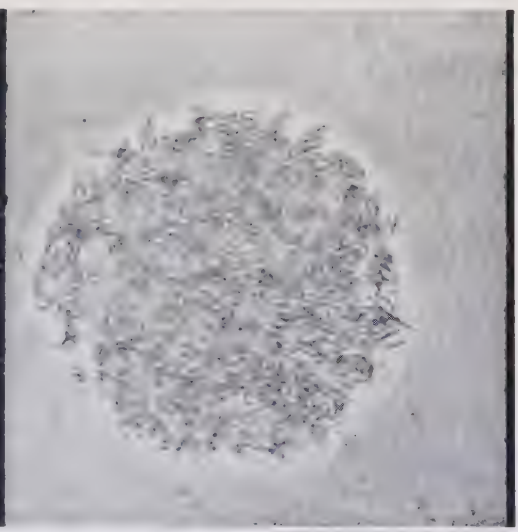

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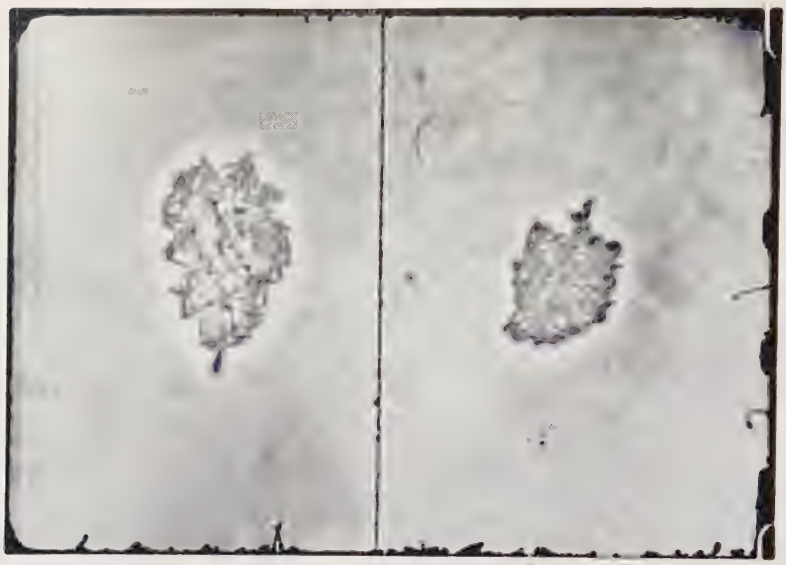
W. H. Hughes — The Advantages and Uses of Phase Contrast Microscopy. Plate 1



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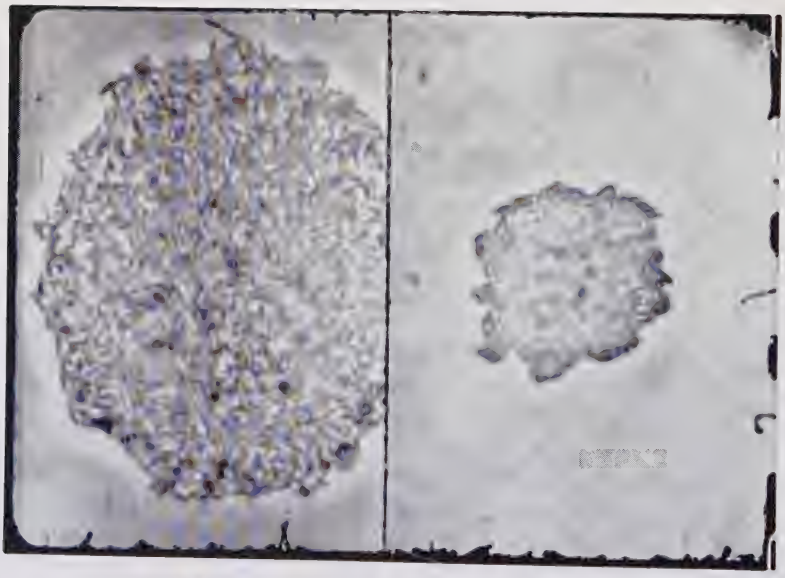

(*c*) (*d*)

W. H. Hughes — The Advantages and Uses of Phase Contrast Microscopy. Plate 2

Teaching films

The results that we have obtained have been cut to give a number of teaching films which are now available from our Photographic Department, and it should be pointed out that while useful scientific information can be obtained quite quickly by this method, a finished film is a very different proposition since loss of focus, a little vibration or irregularities in the background will be offensive when shown to critical audiences. We find that after the necessary photography has been completed, a ten minute film will require up to a year for editing, titling and recording. However, we have here a method which will make available for teaching, material which could not otherwise be recorded.

Examples are given in Plates 1 and 2, which show pairs of frames from our films. In Plate 1 the looped organism in (a) has ruptured in (b) and discharged its contents, which are still visible in (c) but have dispersed in (d), leaving a colony in which it would be impossible to tell that any abnormal cells had been present. Plate 2 illustrates the effect of chloromycetin on the rate of growth of *Klebsiella pneumoniae*.

Explanation of Plates

Plate 1. *Proteus vulgaris*. Pairs of frames separated by 3 min intervals. The looped organism in (a) has burst and discharged its contents in (b). The cell contents are still visible in (c) but have dispersed in (d).

Plate 2. *Klebsiella pneumoniae*. Paired frames, separated by a 4 hr interval, showing the normal development of a colony on nutrient agar at (a) and (c), and at (b) and (d) the restricted development when chloromycetin is present in the medium.

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CONCENTRATION AND MASS MEASUREMENTS IN MICROBIOLOGY

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Contents

		page
1.	Introduction	146
2.	Basic principles	146
	(a) Refractive index and concentration	146
	(b) Principles of measurement	147
3.	Cell refractometry	149
	(a) Bacteriology	150
	(b) Mycology	152
	(i) The mature spore	152
	(ii) Maturation	152
	(iii) Pre-germination and germination	153
	(iv) Spore morphology	154
	(v) The mycelium	155
4.	Cell interferometry	156
	(a) The growth of yeasts	156
	(b) Measurements on bacteria	156
5.	Conclusions	157
6.	Summary	157
7.	Acknowledgements	158
8.	References	158

1. Introduction

A knowledge of the dry mass or solid concentration of living cells provides a valuable basis for much work in quantitative cytology. The usual methods of mass determination, such as the measurement of the wet and dry weights of a culture, only provide crude average figures and give no indication of the range of values to be expected among different members of a population. Within the last few years two methods capable of providing information of this type have become available. We shall discuss here some aspects of these methods as they have been applied to microbiological problems. More detailed accounts of some of these subjects will be given elsewhere.

2. Basic Principles

(a) *Refractive index and concentration*

It has long been known that there is a general relationship between the refractive index of a solution and its content of dissolved substance. Experimental investigations have shown that provided the concentration C of solute is measured on a weight/volume basis (usually in g/100 ml of solution) the relationship is a linear one expressed by

$$n = n_0 + \alpha C \qquad (1)$$

where n is the refractive index of the solution, n_0 that of the solvent and α is a constant, characteristic of the solute, known as the *specific refraction increment*. Thus α is the amount by which the refractive index of the solution increases for an increase of concentration of 1 g/100 ml. It is thus evident that if methods for determining the refractive index of a living cell or some quantity related to it could be developed, information about the solid concentration could be obtained. There is one obvious difficulty, namely that the value of α varies from one substance to another. On further investigation, however, this variation was found to be of minor importance so far as most cellular constituents are concerned. Thus by far the greatest proportion of cell solids is composed of proteins and the value of α for nearly all simple proteins is $0{\cdot}00183 \pm 2\%$. Pigmented proteins often have slightly higher values and lipoproteins slightly lower values, but even so the deviation from the mean is only $\pm 5\%$. Nucleoproteins and nucleic acids, as well as many protein breakdown products, also have values within this range. Carbohydrates have somewhat lower values, namely 0·00143 for the simple sugars and 0·00150 for glycogen, but the quantity is small in comparison with that of proteins. The non-specificity of α may seem strange at first sight, but it must be remembered that the refractive index depends primarily on the number and type of solute atoms and only secondarily on how they are arranged. Most organic substances are composed of much the same sorts of atoms and by the time macro-molecular dimensions are reached, many of the effects of differences in chemical constitution are obscured; for example, the substitution of one amino acid for another in a protein makes virtually no difference to the refractive index. This non-specificity of refractive index is an advantage however, for it enables us to deduce the *total* solid concentration, though of course it tells us nothing about the different types of solutes present. From a consideration of the general composition of protoplasm it has been suggested that a value of 0·0018 be taken for α in living cells (Barer & Joseph, 1954; Davies *et al.* 1954) and this value has been generally accepted.

(b) *Principles of measurement*

The problem now becomes that of measuring the refractive index or some function of refractive index of a microscopic object. Fortunately two sensitive methods exist for this purpose, namely phase-contrast and interference microscopy. The optical details of these methods have been discussed elsewhere (Barer, 1955*a*,*b*, 1956*a*, 1958; Davies *et al.* 1954; Osterberg, 1955) and need not be repeated here. It is sufficient to state that both methods are identical in principle and convert variations in the refractile properties of the object into variations in light intensity. Phase-contrast is actually a form of interference microscopy, but because of constructional peculiarities there are usually certain important differences in the appearance of the image. The fundamental property of the object on which both methods depend is the optical path difference or phase change φ which is defined by the expression

$$\varphi = (n - n_m)t \qquad (2)$$

where n is the refractive index of the object, n_m that of the surrounding medium and t the object thickness. The intensity at any point in the image is a function of φ, though not unfortunately a very simple one. The relationship between intensity

and φ is rather more complicated in phase-contrast than in interference microscopy and whereas it is possible to measure φ with certain interference microscopes, this is not normally possible with phase-contrast. This does not mean that the phase-contrast microscope cannot be used for quantitative measurements as is sometimes stated. On the contrary, it can be used as a very simple and sensitive null point detector for measuring refractive index. From the definition of φ it follows that when the refractive index n_m of the medium is made equal to that of the object, n, φ becomes zero no matter what the value of t may be. The object then becomes virtually invisible. If n_m is just less than n, φ is positive and the object will appear dark by positive phase-contrast (bright by negative phase-contrast). If n_m exceeds n, φ becomes negative and the object appears with reversed contrast, i.e. bright by positive phase-contrast. Thus by immersing a living cell in media of different refractive indices until its visibility becomes minimal, one can measure its refractive index and hence from equation (1) its solid concentration C. The fact that the method is a 'null' one confers certain important advantages. One does not need to measure an absolute quantity but simply to determine whether the cell is bright, dark or invisible. The eye is particularly well adapted for this and can be used with a very sensitive detector (in the form of a phase-contrast objective with a heavily absorbing phase plate). Moreover in some applications, as will be discussed later, one can obtain statistical information on a large cell population by estimating the relative proportion of bright or dark cells in media of different refractive indices. This is of course of particular importance in microbiology.

The interference microscope can also be used as a null point detector in the same way as phase-contrast, but its greater complexity and the need for frequent re-adjustment make it far less convenient. Its main advantage is that it can be used for the absolute determination of φ (for methods of measurement see Barer, 1956*a*, 1958; Davies *et al.* 1954; Osterberg, 1955). At first sight the fact that φ depends on both refractive index and thickness seems to limit its usefulness, for the accurate determination of the thickness of a microscopical object is often very difficult. It can easily be shown, however, that φ can be related to the dry mass/unit area of the cell (Barer, 1952, 1956*a*,*b*; Davies & Wilkins, 1952).

Let us suppose that φ is measured with the cell immersed in water or a dilute salt medium of refractive index n_m. We can write equation (1) in the form

$$n = n_m + \alpha C.$$

On combining this with (2) we obtain

$$\varphi = \alpha C t \tag{3}$$

Multiplying each side by the projected cell area A and expressing C as g/100 ml gives

$$\varphi A = 100 \alpha A t C.$$

But AtC is simply the product of the cell volume At and the solid concentration C, i.e. the total dry mass M; therefore

$$\varphi A = 100 \alpha M$$

or

$$\frac{M}{A} = \frac{\varphi}{100\alpha} = \frac{\varphi}{0{\cdot}18} \tag{4}$$

In other words φ is directly proportional to the dry mass/unit area. This is an

important result, but it must be remembered that whereas the total dry mass of a cell may be a very useful quantity, the dry mass/unit area is only of limited value since it depends on local thickness. In an irregularly shaped or heterogenous cell the total mass can only be found by integrating the product of φ and area over the entire projected area of the cell. This can be done only in very favourable cases, or by means of complicated integrating devices which are not altogether satisfactory (Mitchison *et al.* 1956; Davies & Deeley, 1956; Svensson, 1957). Thus, whereas refractometry is particularly useful for dealing with large cell populations, interferometry only gives results for one cell or even part of one cell at a time. Both methods suffer from certain limitations where intracellular granules or inclusion bodies are concerned. Refractometry can only be applied to the peripheral (cytoplasmic) regions of a cell which are in actual contact with the surrounding medium; one can tell whether an inclusion body has a higher or lower refractive index than that of the cytoplasm, but it is not possible without additional information to obtain the exact value of its refractive index. Similarly in the case of interferometry one can measure the total mass/unit area at a given region, but one cannot determine the contribution of an intracellular body to this mass without knowing the relative thickness of the inclusion and of the surrounding cytoplasm (Barer & Joseph, 1955*b*; Barer & Dick, 1957).

It should be noted that a knowledge of the solid concentration and of the dry mass enables the water concentration and the wet mass to be derived. The water concentration is not simply 100—C because 1 g of protein does not occupy 1 *ml* in solution. A more correct expression for the water concentration is 100—vC, where v is the specific volume of protein, which may be taken as 0·75. (For a summary of some of the formulae used in this field see Barer, 1956*b*).

3. Cell Refractometry

The optical problems involved in measuring the refractive index of a cell have been indicated above. The biological problem is to find a suitable immersion medium. Such a medium must of course be non-toxic but it must also fulfil certain other conditions. Its refractive index must be capable of continuous variation over the range of values likely to be encountered in living cells. It must have a low osmotic pressure so that its tonicity can be adjusted to suit the particular cells examined. If the cells were to shrink or swell in response to osmotic variations in the environment their solid concentration (and hence refractive index) would be affected. This is a very important point in the case of animal cells, but micro-organisms are often much less affected by moderate variations in the osmotic pressure of the medium. Refractometry can in fact be used to study the response of living cells to osmotic changes. It has the great advantage that the cell volume need not be known, as C is inversely proportional to the cell volume (see Dick & Lowenstein, 1958). Finally, the medium must not penetrate the cell, for if it did one could not get a match between the internal and external refractive indices. An accidental observation (Barer & Ross, 1952) led to the realization that these requirements could best be met by solutions of a macromolecular substance such as a protein. Such a substance would have a low osmotic pressure and would be less likely to penetrate the cell

membrane than would a small molecule. In practice the best material so far available is bovine plasma albumin fraction V (Armour Laboratories, Hampden Park, Eastbourne, Sussex) which can be obtained commercially as a dry powder soluble up to concentrations of about 55%. This protein has been found suitable for use with all types of cells so far examined. Although many other macromolecular substances have been tested, only one, namely acacia gum, in any way rivals bovine plasma albumin (Barer & Joseph, 1955*a*). Unfortunately, while acacia gum is quite suitable for use with fungi and some animal cells it is useless for bacteria as it appears to penetrate them, so that no matter how high a concentration is used they never become invisible or appear with reversed contrast. There is evidently a fundamental difference between the behaviour of the cell walls of fungi and bacteria which may be related to differences in their chemical composition.

The immersion method can be used to determine the refractive index of a single cell, provided that the latter can be kept more or less stationary while the medium is changed. This is fairly easy in the case of fungal mycelia but may be extremely difficult for bacteria or fungal spores. In the latter cases therefore a statistical method is used. A minute volume of the bacterial or spore suspension or a tiny scraping from an agar culture is mixed with a small droplet of albumin of known refractive index and then examined by phase-contrast. The refractive indices (and solid concentrations) of the individual cells will be distributed over a range of values. If the concentration of the immersion medium happens to coincide with the modal concentration for the population, many of the cells will be almost invisible, some will appear dark and others bright. If the process is repeated with a slightly more concentrated medium, more cells will appear bright and fewer dark. As the protein concentration is raised still further, the relative proportion of bright to dark cells increases until eventually all the cells appear bright. This gives the upper limit of the range. Conversely, if the protein concentration is decreased, the proportion of bright cells decreases until eventually no bright cells are seen. The concentration then corresponds to the lower limit of the range. Thus, the concentration distribution curve for the whole population can easily be determined by estimating the relative numbers of bright and dark cells in media of different refractive indices. This is not a difficult procedure since one does not have to measure the *degree* of brightness or darkness of an individual cell. In many cases sufficient information is obtained by estimating the limits of the range and the modal value.

(a) *Bacteriology*

We have applied the 'population curve' technique to many species of bacteria and the main results are summarized in Table 1. Where available, concentration values for the range and the mode are given and the results for cultures on solid and liquid media have been compared. As a rule there is not much difference between the values for solid and liquid media, but occasional large differences have been observed (e.g. *Streptococcus haemolyticus* and *Corynebacterium diphtheriae* type *gravis*). The results for an individual strain grown under standard conditions are remarkably constant and the same modal values have often been obtained after intervals of many months. Apart from the differences noted in the Table, the values for a fully grown

culture do not seem to depend very much on the conditions of growth. The final values appear to be much the same whether the medium is a poor or a rich one, provided that it supports growth. Complex changes do, however, occur during the early stages of growth, particularly in the logarithmic phase, but these require further investigation.

Table 1. *Solid concentration (g/100 ml) in various species of bacteria as determined by immersion refractometry*

Species	Solid medium		Liquid medium	
	Range	Mode	Range	Mode
Staphylococcus aureus	36·3 – 40·1	38·5	38 – 40·1	—
Staphylococcus albus	38 – ?	40	38 – 40	—
Streptococcus haemolyticus	? – 35·8	30·5	35·8 – ?	40·1
Streptococcus faecalis	31·2 – 37·8	33·6	? – 33·6	31·5
Streptococcus pneumoniae	33·3 – 39	36·3	27·5 – 39	30·5
Sarcina lutea	30·5 – 33·6	31·5	27·5 – 35·8	31·5
Micrococcus tetragenus	—	—	32·8 – 44·8	39·7
Neisseria meningitidis	23·5 – 36·3	—	31 – 44·6	34·1
Neisseria catarrhalis	—	30·5	21·6 – 28·7	26·5
Salmonella typhi O (X)	24 – 31	28·5	? – 31	29
Salmonella paratyphi B	29 – 31	30	—	31
Salmonella enteritidis	—	—	22·1 – 28·5	23·5
Shigella shigae R	26·5 – 30	29	—	29
Shigella shigae S	? – 29	26·5	—	26·5
Escherichia coli	—	27·5	23·5 – 28·7	25·5
Proteus vulgaris	? – 30·5	26	22·2 – 28	25
Vibrio cholerae	? – 26·5	24·5	—	24·5
Pasteurella muriseptica	23 – 34·6	30	34·6 – 40	36·8
Pseudomonas pyocyanea	—	—	20·6 – 28·5	24·5
Corynebacterium diphtheriae type *mitis*	31·5 – 39	33·1	—	33·6
Corynebacterium diphtheriae type *intermedius*	20·6 – 31·5	25	23 – 36·8	31·5
Corynebacterium diphtheriae type *gravis*	20·6 – 31·5	25	—	34·1
Corynebacterium xerosis	33·8 – 44·8	40·8	36·0 – ?	40·3
Actinomyces bovis	26 – 40·6	34·6	—	—
Mycobacterium phlei	—	40·1	—	—
Clostridium welchii	29·5 – 36·8	31	26·5 – 31	29·5
Bacillus anthracis	? – 27·5	26	23·5 – 31·5	27·5
Bacillus subtilis	30·5 – 35·8	33·6	27·5 – 31·5	29·5
Bacillus cereus	—	—	24·7 – 32·8	28·7
Serratia marcescens	26·2 – 28·7	27·7	27·7 – 31·8	—
Chromobacterium violaceum	23·8 – 32·8	26·2	—	—
Rhodospirillum rubrum	—	—	37·1 – 47·1	43·7
Rhodopseudomonas spheroides	—	—	28·2 – 31·8	29·7
Rhodopseudomonas palustris	—	—	28·7 – 32·8	29·7
Rhodopseudomonas capsulatus	—	—	32·8 – 34·9	33·8

As will be seen from the Table the majority of bacteria so far examined have a solid concentration of the order of 30–40%. This is quite high as compared with the cytoplasmic concentration of most animal cells, which is usually in the range 10–25%. No obvious correlation between solid concentration and bacterial morphology or type can be observed, though there is possibly a tendency for low values to appear in some Gram-negative organisms. The only other attempt to measure the refractive indices of bacteria seems to be that of von Angerer (1923). He attempted to embed bacteria in various media but found that crystalloid solutions were unsuitable because of their high osmotic pressure and the only protein which he tried, gelatine,

could not be made sufficiently concentrated and was awkward to work with because of its viscosity. He therefore compared the appearance of bacteria in water with emulsions containing droplets of various liquids such as benzene, in water. The best match was obtained with amyl alcohol (refractive index 1·406), but even this appeared to be slightly more refractile than the bacteria. He therefore concluded that the refractive index of bacteria was rather less than 1·40. Considering the crudity of his method, this result is in surprisingly good agreement with those we have obtained. A refractive index of 1·40 corresponds to a solid concentration of just about 40%. Primarily, von Angerer was interested in the turbidity of bacterial suspensions, which is a result of the high refractive index of bacteria. The difference in refractive index between the aqueous medium and the suspended bacteria results in considerable scattering of light, so that such suspensions will behave like a piece of ground glass and may be completely opaque. If, however, the modal refractive index of the culture is determined by the immersion method and a suspension is made in a protein medium having this refractive index, scattering is greatly reduced and the suspension may become almost transparent. This technique is useful for studying the absorption spectra of bacterial pigments *in situ*. A clarified suspension of pigmented bacteria can be treated in the same way as an ordinary solution and its absorption spectrum determined in a routine spectrophotometer. Such studies are particularly necessary in cases where the absorption spectrum of the pigment is changed during extraction, as happens with many photosynthetic pigments. Chlorophyll and bacterio-chlorophyll are probably present in the living cell in combination with proteins. Only the pigment fraction is extracted by the usual solvents and very large changes in the absorption spectrum may result. The clarified suspension technique gives particularly good results with bacteria but can also be applied to other forms of free cells such as algae. Further examples of the applications of this method have been given elsewhere (Barer, 1955*b*; see also Rabinowitch, 1956).

(b) *Mycology*

(i) *The mature spore.* Measurements of solid concentration have been carried out on the spores of many fungal species, particularly in the Phycomycetes and the genus *Penicillium*. The mature spore has one of the highest concentrations of solids found so far in any living cell; in some cases the value exceeded 55% and could not be measured by refractometry. In the majority of cases the modal values lay between 45 and 55%. Detailed Tables of values will be given elsewhere.

(ii) *Maturation.* It was observed in a number of cases that samples of spores from fresh cultures gave consistently lower values than similar samples from old cultures. Further investigation confirmed this impression. Thus spores of *P. roqueforti* gave a modal value of 44·8% when taken from a fresh culture; this culture was then kept in a stoppered bottle for nine weeks, at the end of which time the modal value had risen to 54·3%. The increase was not often as large as this in all species, but it was nevertheless always greater than the limits of experimental error. Since the culture bottles contained visible droplets of water, it seems probable that the changes were due to a natural process of dehydration or syneresis with age and not to simple drying or evaporation.

Striking examples of changes in solid concentration during spore maturation are seen in the conidia of some *Penicillium* species. Long chains of conidia are often seen attached to the intact conidiophore; the distal conidia are the most mature. Plate 1 (a) shows such a chain of conidia from *P. cyclopium* immersed in a 37% albumin solution. The youngest conidia have the same solid concentration as that of the sterigma (about 27%) and appear bright, but the concentration increases progressively along the chain so that the conidia become less bright, then almost invisible, and towards the end of the chain, where the concentration exceeds 37%, they appear dark. One can, of course, vary the point at which the changeover from bright to dark occurs by altering the concentration of the medium. Thus in Plate 1 (b) a single conidium of *P. notatum* is seen attached to the conidiophore. The concentration of the medium is 32%, which is higher than that of the bright conidiophore but lower than that of the dark conidium which is beginning to mature and contains a pale nuclear region.

(iii) *Pre-germination and germination.* If a fresh suspension of mature spores is examined in a medium containing 35–40% of protein or acacia gum, all the spores will usually appear dark by positive phase-contrast. Some hours later, however, it may be found that some of the spores are pale or even bright (Plate 1, c and d). Later still germinal tubes begin to emerge from the bright spores. No such tubes ever emerge from a dark spore and the change from dark to bright under these conditions appears to be an essential preliminary to germination. It is thus possible to predict which among a population of spores are going to germinate, often many hours before the germinal tubes appear. In the early stages the change in refractive index is the only distinguishing feature. Later, however, there may be obvious swelling, though this varies greatly in different species. It should be pointed out that the measurement of refractive index is three times as sensitive as the measurement of cell diameter as an index of swelling. Thus an increase in diameter of only 1% in an object as small as a fungal spore would be quite unmeasurable but it would produce a volume change and hence a concentration change of 3%, which would be quite easy to measure. The fall in refractive index or solid concentration is just what would be expected on general grounds, since germination must presumably be preceded by the uptake of water. It is possible that in the early stages this water enters the spore but little change in volume occurs; it may simply fill up the interstices between relatively dehydrated protein structures. Later, however, the spore proteins become more hydrated and swelling occurs. Similar changes can be observed during the uptake of water by some dried proteins and other macromolecular substances. Another possibility which deserves to be investigated is that some solid matter is lost to the medium and replaced by water, as has been shown to occur in the case of some bacterial spores (Powell & Strange, 1953).

By the time the germinal tube has appeared, the mean solid concentration of the spore has fallen to between 20 and 30%. Thus in *Piptocephalis* the value in the resting spore is about 54% but this falls to between 29 and 32% at germination. The corresponding figures for *Zygorhyncus* are 45% and 22–24%. The cytoplasm of the young germinal tube has very nearly the same solid concentration as that of the pre-germinal spore. As the tube grows out, vacuoles appear in the spore

and proximal region of the germinal tube and the solid concentration of these structures falls. The concentration at the advancing tip of the young mycelium usually remains high, however, and more or less constant in value.

Powell (1957) has described changes in the appearance, under phase-contrast, of bacterial spores during germination. He does not give the refractive index of the suspending medium but it must almost certainly have been low. The resting spores appeared to have a dark outline and a bright centre. Before germination the whole of the spore became dark and then began to swell. These changes are due to the same cause as those which occur in fungal spores, namely a fall in refractive index. The bright appearance of the resting bacterial spore is due to an optical characteristic of both phase and interference contrast, namely that when the phase change φ produced by the object exceeds a certain critical value, the object intensity becomes greater than the background intensity. This effect is quite different from the reversal of contrast due to immersion in a medium of higher refractive index (when φ becomes negative) and has been called 'false reversal'. A full discussion of this effect is given by Barer & Joseph (1955*b*). Objects such as spores which have a high refractive index are particularly liable to show false reversal when viewed in a medium of low refractive index such as water. Whereas true reversal can be used to measure refractive index, false reversal cannot and indeed only gives information about the characteristics of the particular phase-contrast objective used. It is much more obvious with objectives which contain heavily absorbing phase plates so that observers who use different microscopes may report different appearances. True reversal on the other hand is quite independent of the type of phase-contrast objective used.

(iv) *Spore morphology.* An important feature of the immersion refractometry method is that it enables the internal structure of a refractile object to be seen with greatly improved detail. Such structure is normally liable to be obscured by false reversal and by the fact that a highly refractile spherical or cylindrical object in a medium of low refractive index behaves like a small lens and disturbs the proper adjustment of the phase-contrast system. If the refractive index of the medium is raised, both the false reversal and the lens effect disappear. This method is thus particularly useful for studying such things as spores, yeasts and some bacteria, as well as spherical animal cells (Barer & Joseph, 1957). If quantitative measurements are not required, concentrated gelatine media can be used in some cases. Excellent results have been obtained with gelatine in the case of yeasts and fungi (Girbardt, 1957; Mueller, 1956), fungal spores (Robinow, 1957) and bacteria (Mason & Powelson, 1956). A surprising amount of internal structure can often be seen in some fungal spores (Plate 1, e and f) and it is clear that much work remains to be done in correlating the appearance of the living spore with the results of staining and histochemical methods. One or more nuclei may be visible as pale vesicular regions usually distinguishable from the much brighter and structureless vacuoles. The latter may be absent in the mature spore, but usually appear in the pre-germinal stage. In some cases, however, as in the spores of *Erysiphe*, vacuoles are always present. The nature of the numerous dark granules, particularly well seen in *Mucor* and *Thamnidium*, is uncertain. Some of these granules stain with tetrazolium compounds; others appear to become rod-like or filamentous when the germinal

tube appears and may be mitochondrial in nature.

(v) *The mycelium.* Spores and mycelia flourish in the protein immersion medium and it has been possible to trace the complete development of the spore and mycelium through germination and the vegetative, asexual and sexual phases. Fig. 1 shows, very diagrammatically, the average concentration of solids in different regions of a mycelium of *Absidia glauca*. It must be appreciated that these values are subject to some individual variation and the diagram is only intended to indicate the general pattern of concentration distribution. The sites of highest concentration tend to be at or near the growing tips of the axial and side branches. Places where active branching is going on also have a high solid concentration. This is particularly true of those regions from which the sporangial initials develop. The sporangium itself has a high value (32%) whereas the sporangiophore and columella have lower values (about 24% at first, falling to 13% or less with maturation). The sexual phase in *Absidia* is particularly interesting. This fungus is heterothallic and the progametangia are developed from two hyphae of different strains, one being designated positive (+), the other negative (−). A striking feature is that the mycelium in the sexual region of the (−) strain has a much higher concentration (15%) than the similar region in the (+) strain (5%). (This has so far been verified in ten out of the twelve strains examined and in the other two exact identification was difficult). Thus in a 10% protein solution one mycelium appears dark and the other bright. In the gametangia the concentration of solids rises and becomes more nearly equal (24% and 17%).

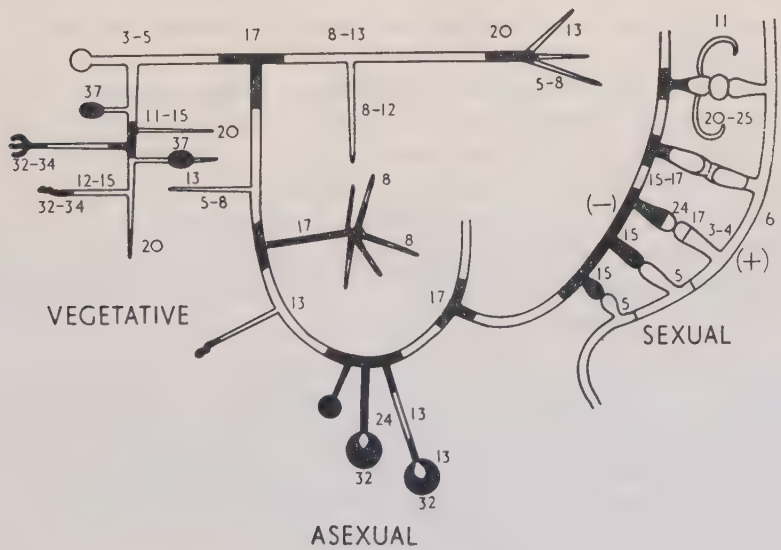

Fig. 1. The variation in solid concentration at different stages of the life cycle of *Absidia glauca*. The values of solid concentration (g/100 ml) are given on the figure and the dark areas indicate regions of high solid concentration. Note the differences in concentration between the (+) and (−) sexual stages. For further explanation see text.

Finally, when the gametangia fuse they contain 20–25% of solids. Similar differences are found in homothallic organisms, e.g. *Zygorhyncus* in which a curved lateral branch grows out from the main hypha. The end of this swells and makes contact with the main branch; gametangia develop and fuse in the region of contact. In this fungus the curved lateral branch has a much higher solid concentration (up to 25%) than the main branch (below 10%). Much remains to be done in order to interpret these findings. A commonly accepted criterion of sex is the greater accumulation of food reserves in the female as compared with the male. On this basis the (–) strain in *Absidia* and the curved lateral gametangiophore in *Zygorhyncus* could be said to have female characteristics. Little attempt appears to have been made to investigate possible chemical differences between (+) and (–) strains. Satina & Blakeslee (1928) analyzed such strains for sugar and fat content but found no important differences. It is possible that any significant difference might be highly localized and would not necessarily be revealed by chemical analysis of a large mass of mycelium. Also, the proteins and nucleic acids may be of greater interest than other substances. The results of refractometry suggest that a full histochemical investigation of the reproductive organs of fungi would be worth undertaking.

4. Cell Interferometry

(a) *The growth of yeasts*

An interesting study has been carried out on the growth of single cells of the fission yeast *Schizosaccharomyces pombe* by Mitchison (1957). For this work he used a photoelectric integrating device to measure the total dry mass of the cell at regular intervals. Volume changes were measured from photographic records. It was found that the dry mass increased more or less linearly throughout growth and cleavage but that the volume remained almost stationary over a period beginning shortly before the initiation of division and ending at cleavage. The average solid concentration, calculated from the total mass and volume, fell to a minimum at the beginning of the constant volume phase and rose to a maximum at cleavage. This is obviously the type of investigation for which interference microscopy is particularly suitable, but it does require rather complex apparatus and the necessary manipulations are by no means easy.

(b) *Measurements on bacteria*

Most of the few microbiological applications of interference microscopy so far reported have in fact been combined with refractometry. Even in Mitchison's work it was necessary for the proper functioning of the integrating apparatus to immerse the cells in a concentrated gelatine medium. Interferometry and refractometry can be combined in two different ways. In the first method the dry mass/unit area (or φ) can be determined by interferometry and the concentration (or n) by refractometry. Since $\varphi=(n-n_m)t$, the thickness t can be deduced. In the second method, φ can be measured at the same point with the object mounted in two different media, for example in water or a dilute salt solution, and in a protein solution of higher refractive

index. If φ_1, φ_2 are the two values obtained, we have

$$\varphi_1=(n-n_{m_1})t \text{ and } \varphi_2=(n-n_{m_2})t.$$

By solving these equations both n and t can be obtained (Barer, 1953; Barer & Dick, 1957). These methods have been applied by Ross & Billing (1957) to the measurement of the solid and water content of bacterial spores. Most bacterial spores have a solid concentration greater than 55%, so that their refractive index cannot be determined by immersion refractometry alone. Ross & Billing used the second method (above) with water and concentrated protein solutions of refractive index about 1·40 as the immersion media. The mean refractive indices of spores of *Bacillus cereus*, *B. mycoides* and *B. megaterium* obtained in this way were 1·512, 1·524 and 1·538 respectively. These values correspond to solid concentrations (g/100 ml) of 99, 105 and 114. Assuming that a completely dry protein corresponds to a concentration of 133 g/100 ml, the water concentrations are 26, 20 and 14 respectively. Bacterial spores must thus be regarded as among the most anhydrous of all living material, considerably more so than most fungal spores, and their refractive indices are of the same order as protein fibres such as wool or leather. For comparison the solid concentrations of the vegetative cells of the three species investigated were found to be 29, 36·5 and 30 respectively.

Ross & Deutsch (1957) have also used a similar method to investigate the shrinkage of air dried bacteria prepared for electron microscopy. In living cells of *Lactobacillus bulgaricus* the mean thickness was 1·16μ by the first method and 1·09μ by the second method. After osmium fixation and drying the thickness was measured by the second method, the two measurements of φ being carried out at first in air and then in water. The mean thickness was now 0·445μ. Measurements with the electron microscope gave the mean width of the cell as 0·475μ. It thus appears as if the cells shrink approximately equally in width and thickness, without appreciable flattening. The degree of shrinkage is quite considerable and indicates the necessity for caution in interpreting the dimensions of specimens in electron microscopy.

5. Conclusions

Both refractometry and interferometry are relatively new methods and no doubt many more applications will be reported in the near future. Each method has its special advantages and disadvantages. Refractometry requires less cumbersome and expensive apparatus and can be used to study large cell populations. Its possibilities for work on cell morphology are also considerable. Interferometry is perhaps more limited in application but can nevertheless sometimes give results unobtainable in any other way. With a proper appreciation of the special uses and limitations of each method the microbiologist is in a good position to increase the scope of his subject by providing much essential quantitative information.

6. Summary

The basic principles of cell refractometry and interferometry are discussed. There is a linear relationship between the refractive index of a solution and the concentration C of solute. Hence the determination of refractive index enables C to be measured

in single living cells. This can be carried out by immersing the cells in media of different refractive indices until a match is obtained between the refractive index of the medium and that of the cell. When this happens the cell becomes virtually invisible under the phase-contrast microscope. This method is particularly well adapted to the study of large cell populations such as occur in microbiology. The interference microscope can be used to measure the optical path difference between a cell and the surrounding medium; this depends on the product of refractive index and thickness and is thus proportional to the dry mass/unit area. Examples of the applications of these methods in various fields of bacteriology and microbiology are given.

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8. References

Angerer, K. von (1923). Die optischen Eigenschaften von Bakterien. *Arch. Hyg., Berl.*, **92**, 14.

Barer, R. (1952). Interference microscopy and mass determination. *Nature, Lond.*, **169**, 366.

Barer, R. (1953). Determination of dry mass, thickness, solid and water concentration in living cells. *Nature, Lond.*, **172**. 1097.

Barer, R. (1955*a*). Phase-contrast, interference contrast and polarizing microscopy. In Mellors, R. C. ed. *Analytical Cytology*. 1st ed. New York: McGraw Hill.

Barer, R. (1955*b*). Spectrophotometry of clarified cell suspensions. *Science* **121**, 709.

Barer, R. (1956*a*). Phase-contrast and interference microscopy. In Oster, G. & Pollister, A. W. ed. *Physical Techniques in Biological Research*. Vol. 3. New York: Academic Press.

Barer, R. (1956*b*). *The Interference Microscope in Quantitative Cytology*. C. Baker: Holborn.

Barer, R. (1958). Phase, interference and polarizing microscopy. In Mellors, R. C. ed. *Analytical Cytology*. 2nd ed. New York: McGraw Hill (in press).

Barer, R. & Dick, D. A. T. (1957). Interferometry and refractometry of cells in tissue culture. *Exp. Cell Res.* Suppl. 4, 103.

Barer, R. & Joseph, S. (1954). Refractometry of living cells. I. Basic principles. *Quart J. micr. Sci.* **95**, 399.

Barer, R. & Joseph, S. (1955*a*). Refractometry of living cells. II. The immersion medium. *Quart. J. micr. Sci.* **96**, 1.

Barer, R. & Joseph, S. (1955*b*). Refractometry of living cells. III. Technical and optical methods. *Quart J. micr. Sci.* **96**, 423.

Barer, R. & Joseph, S. (1957). Phase-contrast and interference microscopy in the study of cell structure. *Symp. Soc. exp. Biol.* **10**, 160.

Barer, R. & Ross, K. F. A. (1952). Refractometry of living cells. *J. Physiol.* **118**, 38P.

Davies, H. G. & Deeley, E. M. (1956). An integrator for measuring the dry mass of cells and isolated components. *Exp. Cell Res.* **11**, 169.

Davies, H. G. & Wilkins, M. H. F. (1952). Interference microscopy and mass determination. *Nature, Lond.*, **169**, 541.

Davies, H. G., Wilkins, M. H. F., Chayen, J. & Lacour, L. F. (1954). The use of the interference microscope to determine dry mass in living cells as a quantitative cytological method. *Quart J. micr. Sci.* **95**, 271.

Dick, D. A. T. & Lowenstein, L. M. (1958). Osmotic equilibria of human erythrocytes studied by immersion refractometry. *Proc. Roy. Soc.* B **148**, 241.

Girbardt, M. (1957). Die Spitzenkoerper von *Polystictus versicolor*. *Planta* **50**, 47.

THE JOURNAL OF APPLIED BACTERIOLOGY

CONTENTS. VOL. 21, No. 1

PAGE

A Polarly Flagellated Member of the Enterobacteriaceae. By KATHLEEN A. COOK 1

Further Observations on the Bacterial Content of Water in Watercress Beds. By LILY A. E. BAKER and EVE BILLING 4

The Taxonomy of the Psychrophilic Meat-Spoilage Bacteria: A Reassessment. By A. D. BROWN and J. F. WEIDEMANN 11

A New Method of Collecting Allantoic Fluid from Embryonated Eggs. By A. KOHN 18

The Nutrition and Some Biochemical Studies of *Vibrio cyclosites* and *Vibrio neocistes*. By W. B. HUGO and MARGARET K. ROGERS 20

A Continuous Infrared Analyser for Measurement of CO_2 in Effluent Air from Bacterial Cultures. By R. C. TELLING, R. ELSWORTH and D. N. EAST . . 26

A New Plug for Culture Flasks. By J. FLIEGELMANN 45

Methods of Assessing the Sporicidal Efficiency of an Ultra-High-Temperature Milk Sterilizing Plant. II. Experiments with Suspensions of Spores in Milk. By J. G. FRANKLIN, D. J. WILLIAMS, HELEN R. CHAPMAN and L. F. L. CLEGG . . 47

Methods of Assessing the Sporicidal Efficiency of an Ultra-High-Temperature Milk Sterilizing Plant. III. Laboratory Determinations of the Heat Resistance of Spores of *Bacillus subtilis* in Water and in Milk. By J. G. FRANKLIN, D. J. WILLIAMS and L. F. L. CLEGG 51

Coli-Aerogenes Bacteria from Farm Dairy Equipment and Raw Milk. By S. B. THOMAS, PHYLLIS M. HOBSON and K. ELSON 58

'Bitty' Cream: The Occurrence and Significance of *Bacillus cereus* Spores in Raw Milk Supplies. By EVE BILLING and W. A. CUTHBERT 65

A Note on the Growth of *Streptococuss bovis* in Thallous Acetate and Tetrazolium Media. By T. F. MEDREK and ELLA M. BARNES 79

Improvements in the Design of a Laboratory Culture Vessel. By R. ELSWORTH, G. H. CAPELL and R. C. TELLING 80

The Failure of Phenol Treated *Escherichia coli* to grow on Membrane Filters. By N. D. HARRIS and J. P. RICHARDS 86

Yeasts Causing Spoilage in Acetic Acid Preserves. By J. C. DAKIN and PAMELA M. DAY 94

The Uptake of Mercury by Seeds Treated with Mercuric Chloride Solution, and its Subsequent Release. By A. H. DADD and S. E. JACOBS 97

A Selective Medium for *Bacillus cereus* in Milk. By KATHLEEN O. DONOVAN . 100

The Rapid Identification of *Escherichia coli* I by the Production at 44° of both Indole and Gas from Lactose. By J. PAPAVASSILIOU 104

Bacteriological Studies of Immersion Cleaning of Milking Equipment on Commercial Farms. By J. W. EGDELL, KATHLEEN L. LOMAX, R. P. ADAMS and MARGARET J. AITKEN 109

The Incidence and Thermal Resistance of Mesophilic Spores found in Milk and Related Environments. By J. D. RIDGWAY 118

Papers read at the Symposium held in London on 8 January, 1958 on New Techniques in Microscopy.

Recent Advances in Electron Microscopical Techniques in Microbiology. By V. E. COSSLETT 128

Ultraviolet Microscopy. By J. SMILES 137

The Advantages and Uses of Phase Contrast Microscopy. By W. H. HUGHES . 143

Concentration and Mass Measurements in Microbiology. By R. BARER and S. JOSEPH 146

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